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# JAPANESE JOURNAL OF OPHTHALMOLOGY

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# PROPHYLAXIS AND THERAPY OF SIDEROSIS RETINAE BY MEDICINES (ATP AND Ca-EDTA)

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Takatsuki City, Osaka

## Introduction

Extraction of iron pieces with use of a magnet has been considered the only treatment for intraocular injuries due to iron pieces, and considerable progress has been made in the locating diagnosis of the pieces and the safe extracting methods.

It is not rare, however, that time is taken between entrance of iron pieces and the extracting operation, and sometimes diagnosis whether or not iron pieces are in the eye is difficult and several days are needed for the diagnosis. Siderosis bulbi develops not seldom or is exacerbated in this interval. Also, it is often experienced clinically that even when iron pieces are extracted with use of a magnet, the inclusion membrane of the pieces is destroyed at the extraction, and siderosis is suddenly exacerbated.

Isn't there any positive means to prevent such intraocular injuries due to iron pieces or cure siderosis which has already developed?

As well known, therapy of retinal degeneration has made great progress owing to rapidly advanced informations on the metabolism and pathology of the retina and introduction of the various relating medicines. Therefore, it is no more a dream to break the wall of the therapy which has been considered unbreakable.

Suggested by these facts, the authors[1,2] started their studies in an unexploited field, prophylaxis and therapy of siderosis by medicines.

Therapy of siderosis may be divided roughly into the following two possibilities.

1) To change iron pieces into harmless substances by chelation of iron ions and discharge them out of the eyeball.

2) To inhibit the harm of iron against tissues.

According to these ideas, in the present study, the authors investigated the effects of so-called metabolism accelerating substances such as ATP and others and a metal chelating agent, Ca-EDTA, through observation on inhibition of development of experimental siderosis retinae.

## Methods

The animals used were adult white rabbits, and 0.25% ferric citrate was used as a tracer of siderosis.

| | | | |
|---|---|---|---|
| Prescription | Ferric citrate | 0.25 | |
| | NaCl | 0.8 | pH 7.6 |
| | Natr. bicarb. | 3.0 | |
| | Aq. dest. | 100.0 | |

This pH and osmotic pressure were the same as those of vitreous humor. This solution, in 0.2 cc.,, was intravitreously injected after suction of the same amount of vitreous humor, and this state was considered experimental siderosis. Various substances shown in Table 1 were used subconjunctivally, subcutaneously or intravitreously in this state, and inhibiting acitons of these substances on siderosis were judged in comparison with controls and normal eyes (Table 1).

Table 1. Substances Used in the Experiments

| Substances | Use and doses | |
|---|---|---|
| | Subconjunctival injection | Subcutaneous injection |
| ATP-Na | 0.5mg. | 5.0mg. |
| Pantothenic acid | 0.5mg. | 1.5mg. |
| Catalin | 0.5mg. | 1.5mg. |
| Thioctic acid | 5.0mg. | 10.0mg. |
| Orotic acid | 10.0mg. | 10.0mg. |
| ATP+Pantothenic acid | | |
| 25% Ca-EDTA | 0.2cc. of 25% Ca-EDTA which was able to chelate the same amount of ferric citrate was intravitreously injected. | |

Twenty-four hours later, the eyeball was taken out after clinical observation, ank fixed in formalin-alcohol. After another 24 hours, macroscopic observations of the posterior eye-cup were comparatively investigated in the field of vision, and then histochemical investigation was made comparatively among the haematoxylin-eosin, PAS, Feulgen, pyronin-methylgreen stained preparations.

Table 2. Comparison of Inhibiting Effects on Siderosis Retnae

| Substances | Effects |
|---|---|
| ATP | ±± |
| Pantothenic acid | + |
| Catalin | + |
| Thioctic acid | + |
| Orotic acid | ± |
| ATP+Pantothenic acid | ±± |
| Ca-EDTA | ±± |

## Results

The results of the experiments were as follows (Table 2).

1. ATP and Ca-EDTA inhibited siderosis retinae remarkably (Figs. 1,2,3).

2. The inhibiting actions of pantothenic acid, catalin, thioctic acid and orotic acid were inferior to those of ATP and Ca-EDTA, and difference among themselves was not definite in the present experiments.

## Discussion

Thus, among biochemical catalyzers, ATP had a remarkable inhibiting action on siderosis, and also Ca-EDTA, a metal chelating agent, showed the marked inhibition.

Then, what is the mechanism of the inhibiting action of ATP on siderosis?

Fig. 1. The retina of a normal rabbit. (PAS stain. ×400)

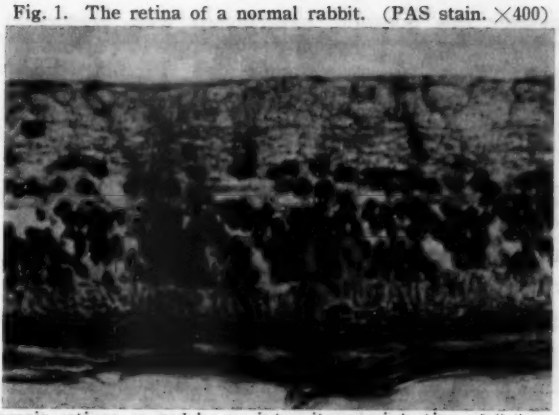


Fig. 2. Siderosis retinae caused by an intravitreous injection of 0.2cc. of 0.25% ferric citrate (24 hours later). (PAS stain. ×400)
Collapse of the outer segment of rods and cones and degeneration and disarrangement of the nuclei of the outer nuclear layer are especially remarkable.

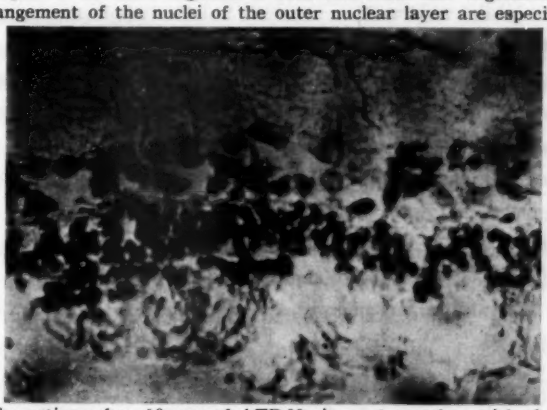
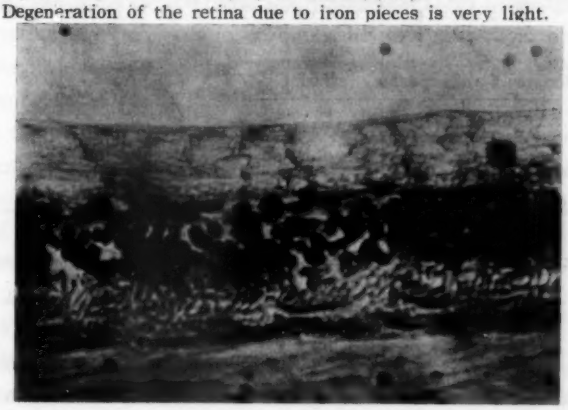


Fig. 3. The retina when 10 mg. of ATP-Na is used together with the causing injection (24 hours later). (PAS stain. ×400)
Degeneration of the retina due to iron pieces is very light.



In order to answer this question, experiments were performed on the following points.

1. Histochemical investigation on the effect of ATP on the normal retina.
2. Influence of hexokinase disturbance due to glyceraldehyde on the inhibiting action of ATP on siderosis.
3. Existence of inhibition of siderosis by a vasodilator (benzyle-imidazolin).
4. Existence of inhibition of siderosis by ADP.
5. Effect of ATP on retinal degeneration due to natrium jodicum ($NaIO_3$).

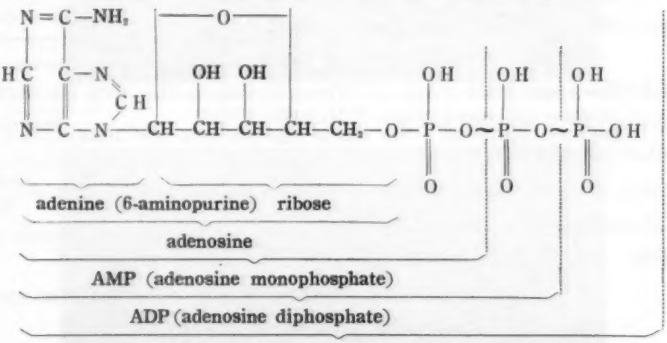
The results were as follows.

1. Vitality of glycogen, DNA and RNA of the normal retina was reinforced by use of ATP.
2. The inhibiting action of ATP on siderosis was almost completely disturbed by M/5000 glyceraldehyde (2.0 cc.).
3. A vasodilator (benzyl-imidazolin) had a very weak inhibiting action on siderosis.
4. ADP did not inhibit siderosis.
5. ATP inhibited experimental retinal degeneration due to $NaIO_3$.

These results suggested that the mechanism of inhibition of siderosis retinae by ATP was due to high energy phosphor molecules in ATP, and the inhibiting action on retinal degeneration was not exclusive to siderosis.

ATP (adenosine triphosphate) is a bound substance of adenyl acid and two high energy phosphor molecules, discovered from muscles by Lohmann[3] in 1928. As well known, it is not only a big source of energy supply, but also an important factor related to the mechanism of composition and resolution in vivo.

Fig. 4. Chemical organization of ATP and its neighbouring substances.



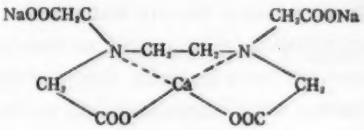

Collins, Love and Morton[4] discovered that regeneration of the visual purple was accelerated by ATP, and various studies have been made, since then, on the relation between ATP and the retina in the ophthalmologic field. It is one of the biochemical characteristics of the retina that unstable organic and inorganic phosphor is abundantly contained in it. It is already obvious that ATP is not only related to the

optic-chemical reaction of the retina but also important and indispensable as an energy source. It is also known that ATP is the energy source for sulfoconjugation.

On the other hand, Cibis[5] expressed his opinion that siderosis was due to disturbance of sulfoconjugation caused by iron. Comparison of this opinion with the results of the present experiments will give rational elucidation.

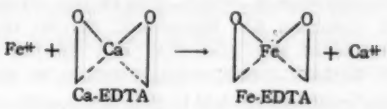
Ca-EDTA (calcium-ethylene diamine tetra-acetate) is a metal chelating agent. It is changed into metal-EDTA in combination with a metal, and has property not to ionize the metal in solution, and thus prevents the toxicity.

Fig.5. Ca-EDTA(Calcium-ethylene diamine tetra acetate).




Mono-Ca-Di-Na-EDTA

Fig. 6. Chelation of iron ions.




It is a significant fact that the inhibiting action of this agent on siderosis, as well as that of ATP, is marked, and this fact suggests that the proper use will contribute to prevention of siderosis.

Besides the inhibiting action, ATP has a considerable recovering action. On the other hand, Ca-EDTA is proved to have no recovering action, and it is natural when the mechanism of the action is considered.

Thus, a clue has been found to positive prevention and therapy of siderosis in the duration between the injury and extraction of iron pieces in which nothing could be done so far, and of enlargement of siderotic foci after the extraction.

Since the cause of siderosis is one and only, the results of the basic studies are considered to be connected directly to the clinical application. In fact, the authors confirmed the effectiveness of application of ATP to cases of intaocular siderosis.

At the present, the authors are using ATP and Ca-EDTA immediately to intraocular perforative injuries due to iron pieces in parallel with the extraction, and obtaining nearly satisfactory results. Needless of saying, the extraction should not be delayed because of the use of these substances.

Judging from the result that ATP was effective not only for prevention and therapy of retinal degeneration due to iron pieces, but also to experimental retinal degeneration due to $NaIO_3$, the authors considered that it might be applied effectively to the other diseases of retinal degeneration. In fact, Oyamada and others[6] have observed considerable effectiveness of ATP clinically to degeneratio pigmentosa retinae, retinitis centralis and others.

## Summary

ATP and Ca-EDTA had marked inhibiting actions on experimental siderosis retinae of rabbits. The mechanism of the inhibition by ATP was rationally explained by inhibi-

tion of sulfoconjugational disturbance by this substance. Inhibition of siderosis by Ca-EDTA was due to the chelating activity. Basing on the results of these basic studies, the authors confirmed the clinical effects on intraocular injuries due to iron pieces.

For the treatment for intraocular injuries due to iron pieces, little more than extraction of the pieces and consideration on infection and disinflammation was done. A great progress has been made toward completion of the therapy by addition of chemical factors to them.

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## THE PATHOGENESIS OF OPTIC NERVE SHEATH HEMORRHAGE REPORT I. OPTIC NERVE SHEATH HEMORRHAGE IN HYPERTENSION

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Hemorrhage of the optic nerve and of its sheath was first described in Japan by Komoto[3] (1897-98) but his was a brief clinical description of a few probable cases of this vascular disorder. Though a similar brief clinical description has been given of it later by several other authors, this ocular disease has never been taken up for systematic investigation by any Japanese author, nor does it seem to attract due attension in current Japanese clininical ophthalmology.

Our pathological examination of the eyeballs and attached optic nerves removed each in its entirety from all available human corpses—corpses offered for pathological dissection—has disclosed that hemorrhage of the optic nerve sheath is a condition of no rare occurrence. This result of our pathological reserch has been confirmed by that of our subsequent daily clinical observation, both pointing to the conclusion that hemorrhage of the optic nerve sheath is recognizable in a considerable proportion of the cases of ocular disease. As a matter of fact, hemorrhage of the optic nerve and of its sheath ascribable to hypertension, trauma, leucemia and other disorders, was noticed in 22 out of those 569 cases in which the eyeballs and optic nerves removed on autopsy were examined during the 5-year period of 1955 to 1959. The following is a description of the pathogenesis of hemorrhages of the optic nerve and the optic nerve sheath due to hypertension observed in 6 out of those 22 cases.

## Materials and Method

The orbital contents were removed from the corpses dissected in the Pathological Department, Kyushu University, in this order:—The brain is removed from the base of the skull; anterior cranial fossa, i.e. the ceiling of the orbit, is cut open with a chisel; the optic canal is cut carefully in the upper wall; the conjunctival fornix is cut along its entire circumference with a scissors; the whole orbital contents, in which the eyeball and the optic nerve are wrapped up, are pushed up from the orbit towards the skull to be extracted intact; the mass of the orbital contents so removed is cast into a 10% solution of formalin and left in it for a few days to be fully fixed; the ocular muscles and the orbital fatty tissues are cut open for the posterior wall of the eyeball and the optic nerve to be exposed to view; the optic nerve cut at the anterior end of the optic chiasm when the brain has been removed from the base of the skull is now observable along its entire length—from its attachment to the eyeball to the posterior end of its canalicular portion, namely to the anterior end of the optic chiasm. Then the optic nerves were each cut at a point 5 to 10 mm. distant from the posterior wall of the eyeball. Macroscopic observation of the retina was made through an opening made at the equator of the eyeball. The optic nerves and eyeballs so treated were embedded separately in celloidin.

## Results

In Table 1 are given the macroscopic findings of the optic nerve and the brain that were presented in 6 cases of hemorrhage of the optic nerve. It will be seen that marked hemorrhage of the optic nerve was accompanied by fatal hemorrhage of the brain in 4 (Cases 1,2,3 and 6) of those 6 cases and that the brain was not macroscopically bleeding in the remaining two (Cases 4 and 5 ), though the clinical record certifies that these two were cases of hypertension while alive. The direct cause of death was evidently post-operative relapse and metastasis into the thoracic cavity of mamma carcinoma in Case 4, and chronic nephritis in Case 5. Histologically, however, the brain was found to have punctately bled in its different parts in these two cases. It is small wonder, though seldom or never pointed out, that nephritic retinopathy and its unfailing accompaniment retinal hemorrhage should have been attended by hemorrhage of the optic nerve in Case 5. Hemorrhage of the optic nerve in nephritic retinopathy is under further histological investigation with us. The ocular fundus was submitted before death to ophthalmoscopic examination in all the six eases but two (Case 4 and 6).

We found that, histologically, hemorrhages of the optic nerve and its sheath occur in four different forms.

1. Epidural hemorrhage.
2. Intradural hemorrhage.
3. Intervaginal hemorrhage.

A. Subdural hemorrhage.
B. Subarachnoid hemorrhage.

4. Intraneural hemorrhage.
A. Pial and septal hemorrhage.
B. Interfascicular hemorrhage.

The histologic findings of the optic nerves and the eyeballs of the six cases are showing in outline in Table 2.

1. Epidural hemorrhage.

Hemorrhage of the epidural tissue was demonstrable along with that of the subdural tissue in 3 (Cases 1, 2 and 3) of the 6 cases. The blood vessels involved in the epidural hemorrhage were most of them veins coming from the connective tissue around the optic nerve and passing through the dura to reach the nerve itself. In these cases the optic nerve was hemorrhagic along its entire length, but the orbital fatty tissue far distant from it was left unaffected, answering in this respect to the description of Ballantyne[1] (1943). This epidural hemorrhage was found to have been produced independenly of the intra- or subdural hemorrhage, neither giving rise to the other in any case.

During our macroscopic observation of the optic nerve in a large number of removed eyeballs from corpses it was noticed in several cases that the nerve itself, though surrounded by bleeding tissuue, was not hemorrhagic in any way. Epidural hemorrhage of the optic nerve is not in itself causative of visual disturbance, and yet, with an extradural hematoma produced along with it, it will indirectly affect vision by compressing the optic nerve. The actual incidence of such advanced extradural hemorrhage among the patients with eye disease remains unknown, but slight epidural hemorrhage may be a condition of no rare occurrence. The arterioles supplying the epidural tissues were markedly sclerotic, some being thrombosed, in 5 out of the 6 cases. The small veins were probably ruptured, for most of the blood shed in this area was found deposited around them.

2. Intradural hemorrhage.

Macroscopically, intra- and subdural hemorrhage was recognizable from the pink-colored or dark-blue-colored specks on the optic nerve. Histologically, intradural hemorrhage, which must, when considered along with Ballantyne's (1943) and Walsh and Hedges'[4](1951)findings, constitute the commonest event that occurred in hemorrhage of the optic nerve sheath, was noted in 5 out of the 6 cases. Intradural hemorrhage was seen in our cases as specks disseminated everywhere in the orbital portion of the nerve and not anywhere in its anterior end portion or in its canalicular portion. Intradural hemorrhage was found continuous with subdural hemorrhage in no small proportion of cases, indicating evidently that the intervaginal space is occasionally involved in intradural hemorrhage. The intradural arterioles were found makedly sclerotic in most of the cases, and intradural hemorrhage was accompanied by intradural leucocytic infiltration in 2 cases (Cases 3 and 6). This leucocytic infiltration was severest where the subdural

hemorrhage was most conspicuous—a fact resulting in all probability from an inflammatory reaction elicited against the blood left stagnant there, though the significance of such an inflammatory reaction remains unknown with us.

3. Intervaginal hemorrhage.

Intervaginal hemorrhage, a phenomenon of most ordinary occurrence during hemorrhage of the optic nerve sheath, was noted either as subdural or as subarachnoid hemorrhage in all our 6 cases. According to Duke-Elder[2] (1947), spontaneous hemorrhage in hypertension is subarachnoid hemorrhage in most cases and not subdural hemorrhage except in rare cases. Our observation of hemorrhage of the optic nerve sheath in 6 cases disclosed that nephritic retinopathy was accompanied by disseminated subdural hemorrhage in one case (Case 5), that subdural and subarachnoid hemorrhage had taken place one with the other in all the remaining 5 cases, and that the subdural hemorrhage was severer than the subarachnoid in 2 (Cases 2 and 6) of those 5 cases, while the former hemorrhage was as severe as, or in some areas less severe than, the latter hemorrhage in the other 3 cases (Case 1, 3 and 4).

A. *Subdural hemorrhage.* This form of blood extravasation may imply either that the blood escaping from the intradural vessels flows ceaselessly into the subdural space or that the blood escaping from the vessels which pass from the dura to the pia flows direct into the subdural space. As will be described later, the wall of blood vessels has its locus minoris resistentiae and is predisposed to hemorrhage in the part where the vessels suffer a sudden change of direction or an alteration of the support afforded by the surrounding tissue, as in the course from the dura to the pia. The subdural space, where there was seen an accumulation of blood, was found to contain in its different parts nodular proliferation of dural endothelial cells.

B. *Subarachnoid hemorrhage.* This was noticed by itself in certain parts of the optic nerve and along with subdural hemorrhage in other parts of the nerve (the latter bleeding occurring alone in certain other parts). It was severest in the frontmost part—more particularly in its temporal side—of the intervaginal space, where the subarachnoid space is broadest. Subarachnoid hemorrhage in the orbital portion of the optic nerve was thus severe in the most anterior part of the nerve and less so in the posterior part. In the posterior part of the optic nerve—posterior to the site of entry of the central retinal vessels—, the intervaginal hemorrhage was widely diversified in severity in different cases; the hemorrhagic focus was extended along the whole length of that part in one case (Case 2), whereas in the remaining 4 cases the amount of shed blood accumulated in the intervaginal space was considerably different in different parts of the nerve, ranging from something to nothing.

The presence of deposited hemosiderin in the hemorrhagic foci showed that the intervaginal hemorrhage had lasted for no small length of time in all these cases. In 3 cases the intervaginal hemorrhagic foci had a number of thickend, sausage-like, hyalinelooking arachnoid trabeculae in them, with nodular proliferation of endothelial cells of the arach-

noid here and there,

The optic nerve is predisposed to subarachnoid hemorrhage in its canalicular portion too. In all the six cases but one (Case 5) the optic nerve had foci of subarachnoid hemorrhage in this part, although in 3 of these 5 cases the optic nerve showed no sign of hemorrhage either in the posterior end division of its canalicular portion or in the optic chiasma, indicating that in these 3 cases subarachnoid hemorrhage of the canalicular part was induced quite independenty of the concurrent subarachnoid hemorrhage of the base of the brain. The subarachnoid hemorrhage of the canalicular portion was also widely varied in severity in different parts of the canaliculus.

4. Intraneural hemorrhage.

Intraneural hemorrhage of the optic nerve may occur in the form of pial and septal hemorrhage or of interfascicular hemorrhage (hemorrhage among the nerve fibers), but practically, of these two induced concurrently. In 2 (Case 4 and 6) of our cases a part of the subarachnoid space in the canalicular portion of the optic nerve, the adjacent pial and septal tissues were occupied uninterruptedly with extravasated blood which penetrated among the nerve fibers too. In another case (Case 3) there were traces of septal and interfascicular hemorrhage alone to be seen in the proximity of the central retinal vessels, i.e. in the axial part of the optic nerve, indicating, presumably, that the blood there came from the vessels supplying the papillo-macular bundle of the nerve. The pia was thickened as a whole with proliferated connective tissue in it. The proliferation of septal glia and connective tissue was particularly conspicuous in the canalicular portion of the nerve, in 4 of the six cases. The small arteries in the pia and the larger septa were markedly sclerotic in all the six cases.

Besides these hemorrhagic lesions of the optic nerve and its sheath, the degenerative changes were observed in the optic nerve. It was seen on Weigert's method of myelin sheath staining that in 4 cases (Case 1, 2, 3 and 4) with marked intervaginal hemorrhage the optic nerve fibers were demyelinating in the peripheral part of the optic nerve, directly under the pia facing the hemorrhagic focus in the subarachnoid space; the nerve fibers were rarefied and each became swollen; the myelin sheath was breaking down into a granular mass or disappeared completely. Towards the center of the nerve, however, the nerve fibers were normally stainable in their myelin sheaths. In 3 cases (Case 1, 2 and 3), the optic nerve was found in its orbital portion to have a few insular areas of ill-staining tissue which were produced, apparently, irrespective of the hemorrhagic foci in the intervaginal space and in the septal tissue.

The lesions of the retina and the choroid of the eyeballs in these 6 cases are enumerated in Table 2: sclerosis of the trunk and branches of the central retinal aretry and of the arteries supplying the choroid; congestion of the trunk and branches of the central retinal vein and of all the veins draining the choroid; papilloedema; localized retinal detachment; hemorrhages of the retinal tissue; sub- and preretinal hemorrhage. Commonest among these lesions was retinal hemorrhage, occurring in all the 6 cases but one

(Case 6). In this case the ophthalmoscopic examination had not been done while alive, but detailed post-mortem histological examination of the eyeballs showed that there was no trace of hemorrhage detectable in the retina.

## Discussion

Among the above-mentioned hemorrhagic disordes of the optic nerve sheath the first to be taken up for consideration is intervaginal hemorrhage. According to Duke-Elder (1943), subdural hemorrhage of the optic nerve sheath is traumatic in origin in most cases, and nontraumatic or spontaneous hemorrhage of it usually occurs as subarachnoid hemorrhage. However, the unacceptability of this view is beyond doubt from the information gained on this problem by Ballantyne, Walsh and Hedge and by ourselves. Subdural hemorrhage of the optic nerve sheath is an event of frequent occurrence even in cases of spontaneous hemorrhage of it induced in hypertension. Intervaginal, and more especially subarachnoid hemorrhage of the optic nerve is invariably more marked in the frontmost part than in any other part of the nerve, undoubtedly because the intervaginal space is widest and ends in a blind alley in this part of the nerve.

Subarachnoid hemorrhage of the optic nerve which is frequently found occurring in the canalicular portion of the nerve, may reasonably be supposed not to have resulted from hemorrhage of the subarachnoid space at the base of the brain on these grounds :—

1. Hemorrhage of the optic nerve sheath was not accompanied by hemorrhage of the base of the brain in some of our cases (Case 4, 5 and 6).

2. In all of our 6 cases except one, the dura, the extradural tissue and the posterior scleral wall of the eyeball were found to have bled concurrently with the intervaginal hemorrhage of the opitc nerve. Needless to add, none of these tissues is in no communication whatever with the subarachnoid space of the base of the brain.

3. In Case 6, the anterior part of the intervaginal space was found to contain a fresh-extravasated blood in it, while a lot of hemosiderin pigment granules were observable in the hemorrhagic focus of the posterior part of the space, indicating apparently that the hemorrhage in the posterior part had occurred far earlier than that in the anterior part.

4. Hemorrhage in the intervaginal space of the optic nerve showed usually marked discontinuity both in the length and in the circumference of the nerve, in the prolonged course like the orbital portion and in a short course like the canalicular portion, as well.

Intervaginal hemorrhage of the optic nerve, far extensive and severer than hemorrhage of the retina in general, was traceable by itself in Case 6, indicatong that the optic nerve sheath is particularly prone to hemorrhage, probably for these two reasons :—

1) Profuse intracranial hemorrhage like cerebral hemorrhage sends up the intracranial pressure, affects the internal pressure of the intracranial veins and venous sinuses, arrests the back flow of blood from the eyeball and the orbit, and may consequently bring about

hemorrhage of the eyeball and the optic nerve sheath.

2) The vulnerability of the vascular system supplying and draining the optic nerve must also be taken into consideration in this connection. In other words the exsistence of locus minoris resistentiae in the wall of the veins in the optic nerve may be held responsible for the hemorrhagic tendency of its sheath. The veins may easily be ruptured in many spots on their course where they are bifurcated, bent sharply, or pass through surrounding tissues quite different from each other in nature, for example, as Ballantyne has pointed out, the central retinal vessels pass from the optic nerve to its sheaths or from the lamina cribrosa to the retina.

To our thinking too, the second-mentioned vascular circumstances seem to account for frequent hemorrhage of the optic nerve sheath. It is possible that a sudden rise in the intracranial pressure resulting, for example, from intracranial hemorrhage, may prevent the venous drainage of the eye and orbit to give rise to a hemorrhage from the veins of the optic nerve. However, hemorrhage of the optic nerve in our case, where no intracranial hemorrhage was traceable, may more reasonably be attributed to the particular condition of the wall of the veins draining the optic nerve. The blood vessels which pass through the orbital tissue, the dura, the intervaginal space and the pia to enter into the optic nerve, are surrounded in their course by tissues widely different from each other in nature, and the consequent sudden change to be produced in the pressure and properties of their blood, as for instance, in leucemia, may often be causative of hemorrhage of the optic nerve sheath.

In one (Case 5) of our cases, a case of nephrotic retinopathy, the orbital portion of the optic nerve was marked with the occurrence of small subdural hemorrhagic foci scattered along it. We have actually demonstrated in this case the reasonable inference that nephritic retinopathy, which is inevitably accompanied by retinal hemorrhage, may naturally result in hemorrhage of the optic nerve, a posterior continuation of the retinal nerve fibers.

## Conclusion

The eyeballs, and attached optic nerves in their whole length, which were removed from 4 cases of hypertension and death from cerebral hemorrhage, one of hypertension and death from postoperative relapse of mamma carcinoma, and another of chronic nephritis and death therefrom, were examined with naked eye and found to be affected by hemorrhage of the optic nerve sheath and of its surrounding tissue. Histological examination of these optic nerves disclosed the pathogenesis of hemorrhage of the optic nerve and its sheath.

I. Hemorrhage of the optic nerve sheath was usually found occurring in the form of intervaginal hemorrhage, which in turn was occurring as subdural and subarachnoid hemorrhage, one equalling the other in incidence and the two developing together in one

Table 1. Macroscopic Findings of the Optic Nerve and the Brain

| Case No. | Name | Sex | Age | Clinical Diagnosis | Blood Pressure | Optic Nerve | Barin |
|---|---|---|---|---|---|---|---|
| 1 | Ito | ♀ | 82 | Hypertension. Cerebral Hemorrhage | 215—105 mmHg. | Left eye: Blue discoloration of the orbital portion of the optic nerve between 3 and 13 mm. behind the globe. Bulbous distension of the anterior end portion of the nerve. Extensive hemorrhage in the fatty tissue around the optic nerve. Marked engorgement and tortuosity of veins in the orbit. Dilatation of small blood vessels on the posterior surface of the eyeball and punctate hemorrhages there. Right eye: Dark blue discoloration of the anterior part of 25 mm. length of the optic nerve behind the globe. No discoloration of the nerve posterior to this part. Otherwise almost similar findings to those of the left eye. | Extensive subarachnoid hemorrhage over the base of the brain. Hemorrhage in the lateral ventricles. Marked arteriosclerosis of the basilar artery. |
| 2 | Shibata | ♀ | 57 | Hypertension. Cerebral Hemorrhage | 200—100 | Both eyes: Extensive dark blue discoloration of the whole length of the orbital portion of the optic nerve, Patchy discoloration in the canalicular portion. Ampullaceous distension of the anterior end portion of the nerve. Extensive hemorrhage in the fatty tissue surrounding the optic nerve. | Hemorrhage into the superficial layers of the frontal lobe and the parietal lobe. Gross subarachnoid hemorrhage over the base of the brain, as well as hemorrhage in the lateral ventricles. Marked sclerosis of the basilar artery. |
| 3 | Mochiyama | ♀ | 68 | Hypertension. Cerebral Hemorrhage | 280—122 | Left eye: Extensive blue discoloration of the whole length of the orbital portion of the optic nerve. Disseminated discoloration of the canalicular portion. Ampullary distension of the anterior end portion. Extensive hemorrhage in the fatty tissue around the optic nerve. Engorgement and tortuosity of veins in the orbit. Right eye: Similar findings to those of the left eye, except no discoloration of the posterior part of the canalicular portion. | Subarachnoid hemorrhage extended from the base of the brain to the cerebellum, as well as marked hemorrhage in the lateral ventricles and fourth ventricle. |
| 4 | Tsuru | ♀ | 65 | Postoperative Relapse of Mamma Carcinoma. Hypertension | 154—70 | Both eyes: Blue discoloration of the whole length of the optic nerve from behind the globe to the anterior part of the canalicular portion. No hemorrhage in the posterior part of the canalicular portion and the optic chiasma. | Macroscopically no hemorrhage in the brain. |
| 5 | Hayashi | ♂ | 21 | Chronic Nephritis. Nephrotic Retinopathy | 200—120 | Both eyes: Patchy discoloration of the nerve throughout its orbital course. | No marked change in the brain. |
| 6 | Yoshikawa | ♂ | 52 | Hypertension. Cerebral Hemorrhage | 208—110 | Both eyes: Disseminated hemorrhagic speck sthroughout the whole length of the optic nerve, from behind the globe to the canalicular portion of the nerve. No hemorrhage in the chiasma and attached portions of the optic nerves. No hemorrhage in the orbital fatty tissue. | Egg-sized hemorrhage in the left temporal lobe. No hemorrhage in the base of the brain. Sclerosis of the basilar artery. |

Table 2. Microscopic Findings of the Optic Nerve and the Eyeball

| Case No. | Optic Nerve | Eyeball |
|---|---|---|
| 1 | Subdural and subarachnoid hemorrhages throughout the whole length of the optic nerve, but with discontinuity of hemorrhage both in the length and in the circumference of the nerve. Epidural and intradural hemorrhages in places of the orbital portion. Demyelinization of the peripheral nerve fibers of the optic nerve. | Papilloedema. Localized detachment of the retina around the optic disc. Hemorrhages in the outer plexiform layer of the retina. Marked sclerosis of the central retinal artery and the choroidal arteries. |
| 2 | Subdural and subarachnoid hemorrhages, the former predominating the latter in number. Subarachnoid serous exudation. Epidural and intradural hemorrhages. Demyelinization of the peripheral optic nerve fibers. | Papilloedema. Preretinal hemorrhage. Hemorrhages in the nerve fiber layer of the retina. Sclerosis of the central retinal artery. |
| 3 | Subdural and subarachnoid hemorrhages throughout the whole length of the optic nerve, in equal proportion of occurrence. Intradural hemorrhage and intradural leucocytic infiltration in some places of the orbital portion. Septal and interfascicular hemorrhages in the axial part of the anterior orbital portion. | Hemorrhages in the nerve fiber layer or the retina. Subretinal hemorrhage. Localized detachment of the retina in the macular region. Marked sclerosis of the central retinal artery and the choroidal arteries. Marked congestion of choroidal veins. |
| 4 | Subdural and subarachnoid hemorrhages in the orbital portion. Disseminated intradural hemorrhage. Pial and septal, and interfascicular hemorrhages in the posterior end part of the orbital portion. No hemorrhage in the canalicular portion. | Hemorrhages in the nerve fiber layer of the retina. Preretinal hemorrhage. Sclerosis of the central retinal artery and the choroidal arteries. |
| 5 | Disseminated subdural hemorrhages in the orbital portion. No subarachnoid hemorrhage. | Typical changes of nephrotic retinopathy. |
| 6 | Subdural and subarachnoid hemorrhages throughout the whole length of the optic nerve. Subarachnoid serous exudation. Intradural hemorrhage and intradural leucocytic infiltration in the orbital portion. Pial, septal and interfascicular hemorrhages in the canalicular portion. | No retinal hemorrhage. Localized detachment of the retina in the macular region. Marked sclerosis of the central retinal artery and the choroidal arteries. Marked congestion of the retinal veins and the choroidal veins. |

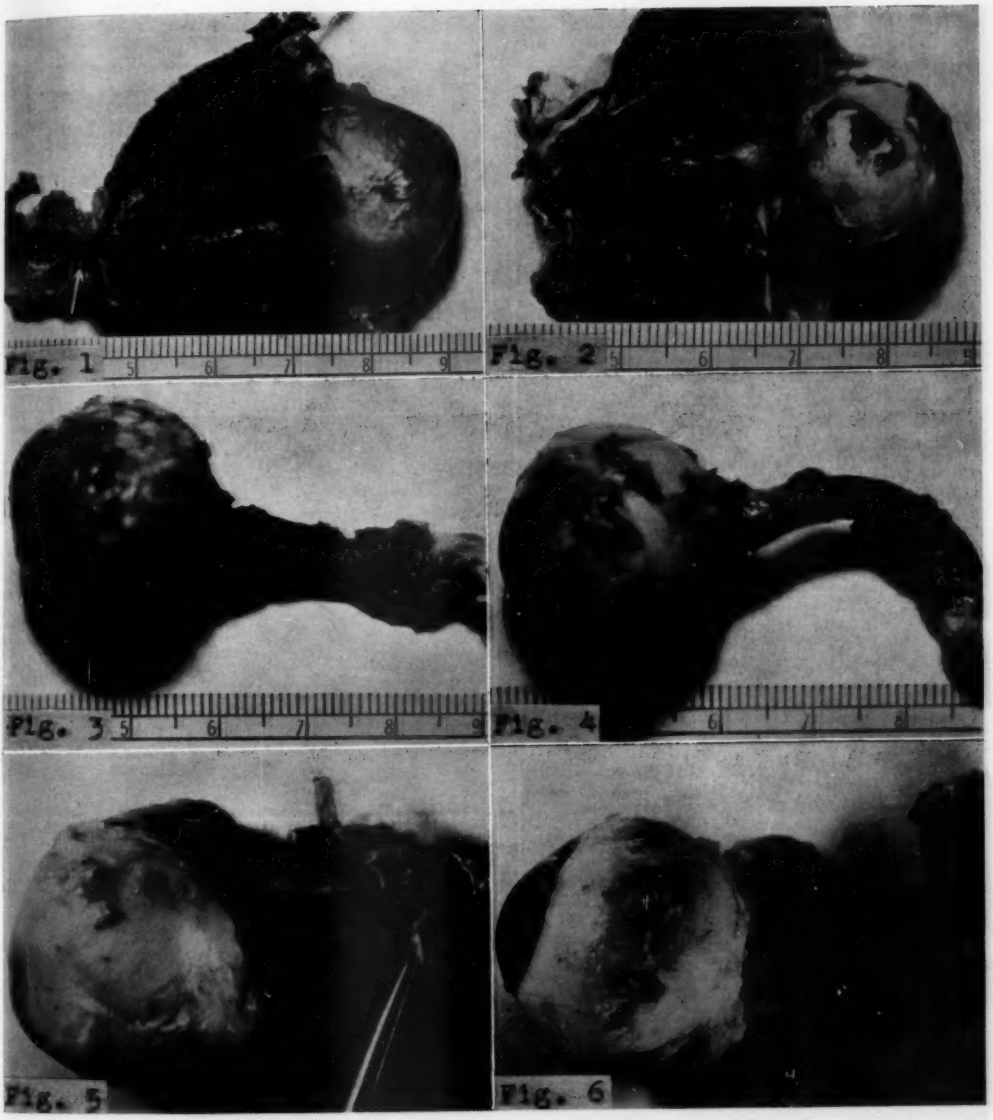
Fig. 1
Fig. 2
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Fig. 6

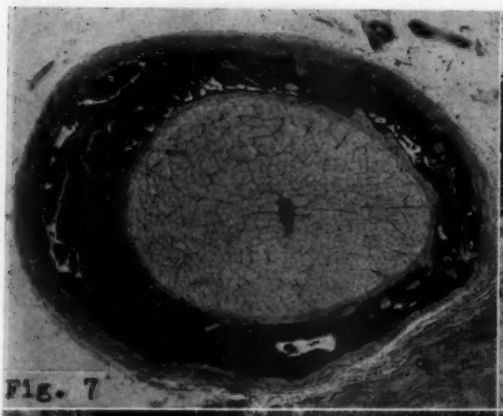

Fig. 7

Fig. 8

Fig. 9

Fig. 10

Fig. 11

Fig. 12

Fig. 13

Fig. 14

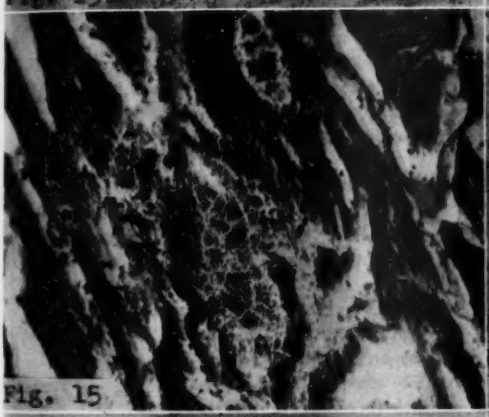

Fig. 15

Fig. 16

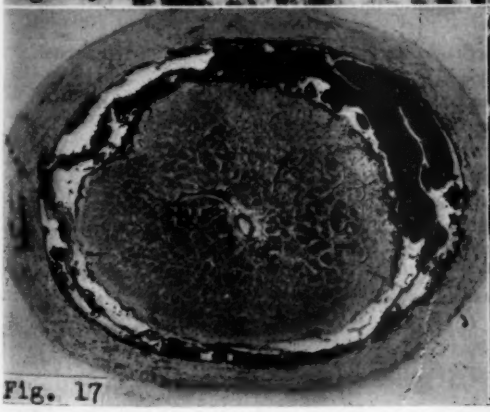

Fig. 17

Fig. 18

## Explanation of Figures

Fig. 1. Left eye of Case 1. There is an extensive hemorrhage in the orbital fatty tissue surrounding the optic nerve. The orbital portion of the optic nerve is dark blue due to its sheath hemorrhage exclusive of the anterior segment of 3 mm. length behind the globe. The veins in the orbit are engorged and tortuous. The small blood vessels on the posterior surface of the eyeball are markedly dilated and a few punctate hemorrhages there. Arrow indicates the canalicular portion of the optic nerve.

Fig. 2. Right eye of Case 1. Similar view to that of the left eye. The anterior end portion of the optic nerve shows an ampullaceous distension.

Fig. 3. Left eye of Case 2. An extensive sheath hemorrhage is seen in the orbital portion of the optic nerve. Disseminated hemorrhages are observable in the posterior end of the canalicular portion of the nerve. The orbital portion behind the globe shows a bulbous distension.

Fig. 4. Right eye of Case 2. Similar view to that of the left eye.

Fig. 5. Left eye of Case 3. A marked hemorrhage is present in the sheath of the whole orbital portion of the optic nerve, but no hemorrhage is traceable backward in the canalicular portion. The orbital fatty tissue surrounding the optic nerve is extensively hemorrhagic. Note the marked dilatation and tortuosity of the veins around the optic nerve.

Fig. 6. Right eye of Case 3. Similar finding to that of the left eye. Hemorrhage is present in the sheath of the whole orbital portion of the optic nerve and can be traced in that of the canalicular portion. The anterior end part of the nerve distends ampullaceously.

Fig. 7. Extensive subdural and subarachnoid hemorrhages in the anterior end portion of the optic nerve. Left eye of Case 1.×14.

Fig. 8. Subdural and subarachnoid hemorrhage in the frontmost part of the optic nerve. Left eye of Case 4.×25.

Fig. 9. Subdural hemorrhage and subarachnoid serous exudation. Hemosiderin pigment granules are noticeable in the hemorrhagic focus. Slight degeneration is occurring in the peripheral nerve fibers beneath the pia. Left eye of Case 2.×70. D: Dura.

Fig. 10. Intra- and subdural hemorrhages and subarachnoid hemorrhage. Thickend hyaline trabeculae of the arachnoid are observable. Left eye of Case 1.×108. D: Dura.

Fig. 11. Intra- and subdural hemorrhages. Arrow indicates an intradural hemorrhage. Left eye of Case 2.×70. D: Dura.

Fig. 12. Intra and subdural hemorrhages. Arrows indicate intradural hemorrhagic foci. Left eye of Case 2.×70.

Fig. 13. Subarachnoid, pial and interseptal hemorrhages in the posterior end part of the orbital portion of the optic nerve. Left eye of Case 6.×48.

Fig. 14. Intraneural hemorrhage in the neighbourhood of the central retinal vessels. Arrows indicate hemorrhgic foci. Left eye of Case 3.×70.

Fig. 15. Interseptal and interfascicular hemorrhages. Left eye of Case 4.×120.

Fig. 16. Subarachnoid hemorrhage and nodular proliferation of arachnoid endothelial cells. Left eye of Case 1.×118.

Fig. 17. Demyelinization of peripheral nerve fibers just inside the marked intervaginal hemorrhage. Arrows indicate the marked demyelinating part of the optic nerve fibers. Left eye of Case 3.×19.

Fig. 18. Demyelinization of peripheral nerve fibers of the optic nerve beneath the pia. Right eye of Case 3.×70.

area or separately in different areas.

2. Subarachnoid hemorrhage of the optic nerve was seen occurring not as sequel to, but independently of, that of the base of the brain.

3. Intervaginal hemorrhage of the optic nerve was often noticed concurrently with intracranial hemorrhage or with subarachnoid hemorrhage of the base of the brain, though it was occasionally traceable when the brain had little or no blood extravasated in it.

4. Intradural hemorrhage was also high in incidence, not infrequently with the subdural space involved in it.

5. Intraneural hemorrhage of the optic nerve was also recognizable in the form of pial and septal hemorrhage and interfascicular hemorrhage combined; the former—pial and septal hemorrhage—was invariably coexistent with subarachnoid hemorrhage, whereas septal and interfascicular hemorrhages were occasionally recognizable by themselves in the axial part of the optic nerve.

6. In one case of nephritic retinopathy were there seen traces of subdural hemorrhage of the optic nerve without any blood extravasated in the brain.

7. In cases of marked intervaginal hemorrhage of the optic nerve the peripheral nerve fibers adjacent to the hemorrhagic focus in the intervaginal space were found to be demyelinated first.

8. Hemorrhage of the optic nerve and surrounding tissues, like that of the retina, is likely to take place of frequent intervals. If so, it would be advisable to keep always in mind the possible occurrence of this bleeding which, unlike retinal hemorrhage, is indetectable under ophthalmoscopic examination.

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## PAPER ELECTROPHORETIC STUDIES ON PROTEINS OF RABBIT OPTIC NERVE TISSUE

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Literature dealing with the biochemical analysis of the visual pathway including the optic nerve is very few. The literature which can be found today is as follows: the metabolism of acetylcholine by Toyoshima, the histochemical studies on phosphatase by Allen and Friendenwald, the metabolism of phosphoric acid with $P^{32}$ by Tani and Kobayashi, tissue respiration and glycolysis by Fujisawa, the glycolysis of the optic nerve involving secondary degeneration by Imachi and others, and a report made by Tani on the metabolism of phosphoric acid and a report of Sugasawa on the histochemistry of nucleic acid by means of radioautography respectively. Furthermore, any of the system-

atic studies concerning the biochemical analysis of the visual pathway have not been presented and even such basic data as the normal chemical composition are not sufficient to warrant any conclusions.

Multiple sclerosis has been considered to be very rare in Japan, but recently it is said that this is not the case and at the same time, the problem of retrobulbar neuritis is now warmly discussed with special reference to demyelinating diseases including multiple sclerosis. From above-mentioned fact, the biochemical studies on the visual pathway must play a significant role in finding the pathogenesis and the appropriate treatment of multiple sclerosis. Very little amount of the tissue material, however, can be available to biochemical analysis of the visual pathway even when large experimental animals such as rabbits and dogs are used. Therefore, it seems generally to be difficult to attempt a biochemical analysis and this is likely to be one of the reasons why biochemical data with regard to the visual pathway, especially the optic nerve are insufficient. The visual pathway including the optic nerve is a part of the cerebrum and belongs to the white matter. Consequently, it seems to be plausible that biochemical properties of the white matter of the cerebrum may be applied also to the visual pathway. It is known, however, that biochemical properties of the white matter of the cerebrum differ to some extent according to its locality. From these point of view, an attempt was made in this paper to study soluble proteins of the optic nerve by means of paper electrophoretic method, compared with that of the brain tissue. This is also a preliminary experiment for studying clinically the electrophoretic pattern of proteins of the cerebrospinal fluid in various kinds of optic nerve diseases.

## Materials

1) The normal rabbits:

Adult white rabbits weighing about 2 kg., which were used after more than 2 weeks feeding with routine diet.

2) The rabbits, the optic nerve of which was experimentally subjected to Wallerian degeneration:

The rabbit eyes were removed after 30 days of feeding with routine diet. It was confirmed that the operated wound gave rise to no infection and the optic nerves were led to Wallerian degeneration.

3) The rabbits with allergic encephalomyelitis produced experimentally:

Brain tissue suspension mixed with Freund-adjuvant which was indicated by Kabat and others, was used for producing allergic encephalmyelitis. i.e.:

a) Liquid paraffin (Sterilization by high pressure).

b) Anhydrous lanolin (This was sterilized by heating at 60°C for 30 minutes once a day for consecutive three bays).

c) Tuberculous bacilli killed by heating (Intermittent sterilization was performed by heating at 100°C for 30 minutes. After that, the suspension was prepared so as to contain

10 mg. wet weight of the bacilli per 1 ml.).

d) As to antigen, the white matter of the brain of normal rabbit excised by sterile manner was used, and the white matter was homogenized by Potter-Elevehjem's homogenizer adding sterilized physiological saline solution.

And then, d) was mixed with the same amount of b)···e). In the meanwhile, the suspension was prepared at the ratio of 10 mg. of c) to 10 cc. of a)···f). Then, e) and f) were mixed up with each other at the ratio 1:1.

The rabbits received a intramuscular or intraperitoneal injection of 0.5 cc. of such brain tissue suspension as described above, and the same amount of the suspension was injected repeatedly 7 and 10 days after the first injection. From 3 to 5 days after the last injection, the rabbits showed signes of encephalomyelitis. The developed symptoms were as follows: motoric paralysis, ataxia, tremor, spinalstiffness, blepharoptosis, anisocoria, feebleness or disappearance of pupillary light reflex,and incontinence.

The order of appearance and the severity or degree of these symptoms differ with cases, but in almost all cases the symptoms developed progressively. The rabbits which had distinctly showed the signs of encepharomyelitis were used as the material for this experiment.

## Methods

The rabbits prepared as described above were killed by air injection into the vein or bleeding from the carotid artery. Craniotomy was made immediately after death and the brain was excised as soon as possible. The excised brain was washed in physiological saline solution, and blood and cerebrospinal fluid were removed as completely as possible. The washed brain was frozen by carbon dioxide and it was smashed into pieces, which were put into a mortar and after being made into a paste vernal-vernal-Na buffer solution ($pH=8.8, \mu=0.05$) was added twice as much as the amount of the brain paste, and this homogenate was kept in an ice-box (O°C-4°C) for 10 hours, and then it was centrifuged at 4,000 rpm. for 30 minutes at room temperature. Thus, the supernatant containing soluble proteins of 0.5 to 1.5% was obtained. This was poured into a cellophane sack and concentrated with a diffusate, 20% polyvinylpyorrolidon, so that the concentration of the protein in the dialyzed solution might be in 5 to 8%. This dialyzed solution was used for paper electrophoretic studies by means of Grassmann-Hannig's method with horizontal paper (Toyo filter paper No.51), and this experiment was performed under the following conditions; 0.25 mA/cm, 200 V, at room temperature, the amount of a sample placed on filter paper 0.02 cc. After an electric current was passed through filter paper soaked in an electrolyte solution for approximately 10 hours, the filter paper was removed from the electrical field and dried in order to fix the protein components in the areas to which they had migrated. The filter paper was then stained with a dye, bromphenol blue, which combines with and staines the proteins. The density of the staining reaction was esti-

mated with a densitometer (Toyo Koden). This paper electrophoretic procedure followed the standard method adopted by Japan Electrophoretic Committee.

## Results

*Consideration of concentration procedures*:

The amount of soluble proteins of the brain (gray and white matter) or optic nerve including optic tract are too small to perform the electrophoresis favorably. Therefore, the material must be concentrated to a certain extent. The amount of soluble proteins extracted in the author's experiment in the normal rabbits has been approximately 0.5% in the optic nerve, 1% in the white matter and 1.5% in the gray matter. The electrophoretic pattern of the proteins could not be distinctly obtained in these rate of concentration of the extract.

The procedure of concentration was done by dialytic method using a cellophane membrane. The dialyzing effect depends on concentration, temperature, humidity, and time etc. Therefore, these conditions have been controlled as much as possible, considered from an importance of reproductivity of experimental results. In addition, in order to make sure whether or not this procedure of concentration makes any change in soluble proteins and influences the experimental results, the rabbit serum diluted with physiological saline solution has been concentrated by the method as referred to already, and paper electrophoresis has been performed, parallel with soluble proteins in the original serum. As a result, percentage values of the protein fractions have been substantially the same in both these samples as shown in Tabel 1, and it has been confirmed that the procedure of concentration have little influence upon soluble proteins in the serum. These percentage values of the fractions are approximately equal to these which has been reported so far (Table 1).

Table 1. Effect of concentration procedure (dialysis with Polyvinylpyrrolidone and cellophane) on paper electrophoretic result.

| | | Alb. | $\alpha_1$ | $\alpha_2$ | $\beta$ | $\gamma$ |
|---|---|---|---|---|---|---|
| Original serum of the normal rabbit. | 8.5 % | 63.3 | 6.5 | 5.9 | 10.8 | 13.5 |
| Concentreted serum after dilution of original serum. | 7.75% | 63.5 | 6.6 | 5.6 | 11.2 | 13.2 |

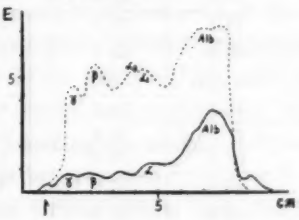
*Percentage values of protein fraction in the normal rabbits*:

In the normal rabbits, five fractions were found to be present in both the brain and optic nerve tissues. The paper electrophoretic pattern of brain tissue extract have been found to be characterized by the absence of a sharp high peak of the fraction corresponding to the albumin fraction which shows characteristically a highest peak in the serum and by the presence of a slack high peak in the range corresponding to the $\beta$-globulin fraction in the serum. The other peaks have not been so distinguished as can be

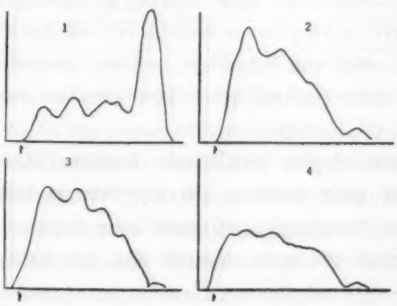
Table 2. Percentage values of fractions of soluble proteins in normal rabbit.

| | P.A. | I | II | III | VII |
|---|---|---|---|---|---|
| Optic nerve (together with optic tract) | 3.9 | 7.7 | 49.0 | 33.2 | 6.2 |
| | 3.5 | 7.4 | 38.5 | 45.2 | 5.4 |
| | 3.8 | 8.1 | 42.1 | 39.9 | 6.1 |
| | 3.6 | 7.7 | 38.5 | 45.0 | 5.2 |
| | 3.1 | 7.9 | 44.4 | 38.0 | 6.6 |
| Mean | 3.6 | 7.8 | 43.8 | 38.9 | 5.9 |
| Gray matter | 1.3 | 12.0 | 51.1 | 26.8 | 8.8 |
| | 2.0 | 8.8 | 43.2 | 41.0 | 5.0 |
| | 1.6 | 9.5 | 38.5 | 46.3 | 4.1 |
| | 1.5 | 9.9 | 40.8 | 39.7 | 8.1 |
| | 1.1 | 11.2 | 45.5 | 37.5 | 4.7 |
| Mean | 1.5 | 10.3 | 43.8 | 38.3 | 6.1 |
| White matter | 2.1 | 17.1 | 41.7 | 31.0 | 8.1 |
| | 3.0 | 7.8 | 44.3 | 38.0 | 6.9 |
| | 2.8 | 12.3 | 34.7 | 42.2 | 8.0 |
| | 2.2 | 9.2 | 42.1 | 39.4 | 7.1 |
| | 1.9 | 8.8 | 39.5 | 41.3 | 8.5 |
| Mean | 2.4 | 11.0 | 40.5 | 38.4 | 7.7 |

Fig. 1. Protein patterns of the serum(···) and normal brain tissue extract mixed with the diluted serum 1:1 (—) in a case of normal rabbit.

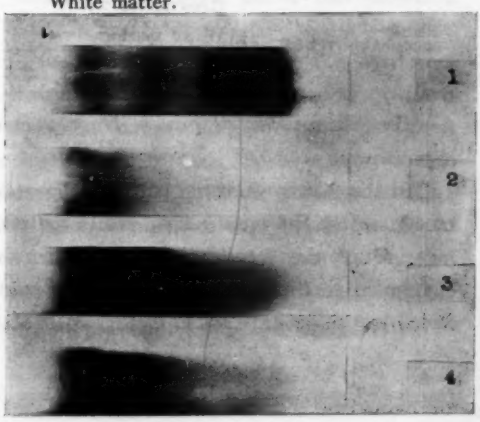



Photoelectlic scanning patterns in the coresponding stained filter paper (right).




seen in the pattern of the serum and cerebrospinal fluid (Table 2, Fig.2). In the meantime, the brain tissue extract mixed with the same amount of the serum diluted 1:1 with the buffer solution has been submitted to paper electrophoresis and this experiment has revealed that the second peak of brain proteins corresponds to a peak of serum albumin and that a component which moves faster than serum

Fig. 2. Electrophoretic patterns on the filter paper in a normal rabbit. 1) Serum. 2) Optic nerve together with optic tract. 3) Gray matter. 4) White matter.



albumin exists in brain proteins (Fig. 1). This component is most likely to correspond to prealbumin which can be found also in the cerebrospinal fluid. Although this only means, however, that in the rate of mobility this component is quite similar to prealbumin of the cerebrospinal fluid, and there is no confirmation that this is the same protein component with that of the cerebrospinal fluid, it has been designated as prealbumin. And the protein fractions on the filter paper, except for prealbumin, have been designated in order of decreasing mobility as the figures I, II, III, and IV which seem to correspond to albumin, $\alpha$-, $\beta$- and $\gamma$-globulins respectively. The subfractions of $\gamma$-globulin have not been identified in the brain tissue extract.

*Percentage values of protein fractions in the rabbits with Wallerian degeneration of the optic nerve*:

The prealbumin fraction has been entirely indistinguished in the optic nerve, white matter and gray matter. In the electrophoretic pattern of the optic nerve, an increase of the fraction II and a slight decrease of the fraction I and III have been recognized, and there has been also the same tendency in the white matter. In the gray matter, no change has been found in each fraction with the exception of the prealbumin fraction (Table 3).

Table 3.

| | | P.A. | I | II | III | IV |
|---|---|---|---|---|---|---|
| Normal rabbit | Optic nerve | 3.6 | 7.8 | 43.8 | 38.9 | 5.9 |
| | Gray matter | 1.5 | 10.3 | 43.8 | 38.3 | 6.1 |
| | White matter | 2.4 | 11.0 | 40.5 | 38.4 | 7.7 |
| | | | Alb | $\alpha_1$, $\alpha_2$ | $\beta$ | $\gamma$ |
| | Liver | | 25.5 | 53.5 | 14.5 | 6.5 |
| | Serum | | 63.2 | 6.6, 5.9 | 10.8 | 13.5 |
| Waller's degeneration of optic nerve | Optic nerve | | 4.4 | 52.0 | 35.9 | 7.7 |
| | Gray matter | | 13.0 | 48.6 | 34.1 | 4.3 |
| | White matter | | 6.6 | 44.8 | 32.5 | 16.1 |
| | | | | | | |
| | Serum | | 48.3 | 7.9, 12.6 | 19.0 | 12.2 |
| Exp. allergic encephalomyelitis | Optic nerve | | 7.6 | 46.6 | 26.6 | 19.2 |
| | Gray matter | | 6.9 | 52.7 | 31.0 | 9.4 |
| | White matter | | 15.0 | 44.8 | 20.0 | 20.2 |
| | | | | | | |
| | Serum | | 44.7 | 9.4, 16.4 | 12.1 | 17.4 |

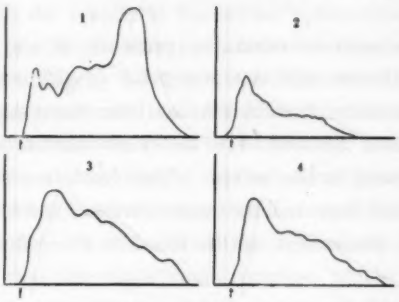
*Percentage values of protein fraction in the rabbits with allergic encephalomyelitis produced experimentally*:

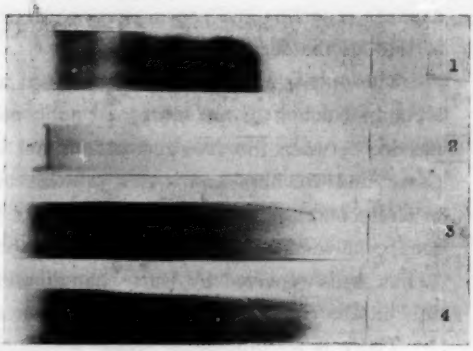
In the electrophoretic pattern, disappearance of the prealbumin fraction has been recognized in the optic nerve, white matter and gray matter. In the former two, an increase of the fraction IV and a decrease of the fraction III have been found. Any change in percentage values of protein fractions of the gray matter has not been produced in this group (Table 3, Fig. 3).

Fig. 3.

Electrophoretic patterns on the filter paper in a rabbit with allergic encephalomyelitis, experimentally produced. 1)Serum. 2)Optic nerve together with optic tract. 3) Gray matter. 4) White matter.

Photoelectric scanning patterns in the corresponding stained filter paper (right).




## Comment

As for the electrophoretic studies on brain tissue proteins, there have been various reports; Kaps (1954), Keep (1955), Hofman and Shinks (1956) Booy (1954), in Japan Homma and Saito (1958), Arima (1958). Paper electrophoretic studies on the peripheral nerves using N. obturatorius of the cow has been reported by Keil. At present, however, the paper electrophoretic procedures are confronted with many still unsolved problems as regard to the ability to separate protein components and reproductivity in the experimental results, and in particular, this fact may be applicable to cases using extract of the tissue including the brain.

*The normal rabbits*:

The normal rabbits have five protein fractions in the optic nerve, gray matter and white matter. The gray and white matters of the brain have shown a plateau-shaped pattern with slack peaks in the fraction II and III which are considered to correspond to $\alpha$-, and $\beta$-globulins of the serum respectively. Meanwhile, the optic nerve has shown a mountain-shaped pattern with relatively sharp peak in the fraction III.

Arima has recognized seven protein fractions in the brain tissue of the dog by means of Tisselius' method and there has been the resemblance in the electrophoretic pattern between the gray and white matters with the exception that the highest peak has been situated at the range of the $\alpha_2$-globulin fraction in the former and at the range of the albumin fraction in the latter. Paper electrophoretic studies has been performed using N. obturatorius by Keil and the protein fractions have been separated very distinctly into four components. These fraction have been designated by Keil as fraction A,B,C, and D, respectively, and the fraction A which is considered to correspond to the albumin fraction in the serum has shown a remarkable high value. In the result of the author's experimen t,

however, though the material used is not the peripheral nerve but the central nervous tissue including the optic nerve, the electrophoretic pattern has been substantially different from that of the serum, notwithstanding that the method of extraction of proteins from the tissue and the experimental conditions have been about the same with those adopted by Keil, and except for his report, a search of the literature has failed to reveal a paper reporting the paper electrophoretic pattern of the tissue extract closely similar to that of the serum.

The author's experiments mentioned above have revealed the presence of the prealbumin fraction in the brain and optic nerve tissues, and this is a point of distinct difference between the reference tissue and the serum. Prealbumin has been suggested by Esser and Bücher, and it is a protein component referred to as faster one than albumin in electrophoretic mobility, which can not be found in the serum. This fraction also has been confirmed to be present in the cerebrospinal fluid and the human brain. In addition, it has been reported by Hara that prealbumin component can be found in the subretinal fluid in the case of retinal detachment.

There is an interesting report to the effect that lipoprotein and nucleoprotein playing an important role in the nervous tissue do not contain a prealbumin component. Its biological significance, however, is quite unknown. From the author's results, the optic nerve, white matter and gray matter have been roughly arranged in order of decreasing parcentage value of prealbumin.

*The rabbits with Wallerian degeneration of the optic nerve.*:

The secondary degeneration may involve the axis cylinder and it may differ from the primary demyelinating process, but because this Wallerian degeneration also results in remarkable destruction of the myelin sheath, the author's experiment will throw some light on the pathogenesis of primary demyelinating process. The Wallerian degeneration has been usually studied by severing of the N. ischiadicus belonging to the peripheral nervous system. The biochemical approach to this problem has been made by Johnson, McNabb, Rosister, et al., and changes in glycolytic system in the dog optic nerve involving secondary degeneration have been reported by Imachi and others. In these reports, the process of secondary degeneration is divided into three stages based upon changes of the myelin sheath.

The 1st stage (lst day to 8th day); Period of destruction of axis cylinder and physical disintegration of myelin.

The 2nd stage (8th day to 32nd day); Period of biochemical disintegration of myelin.

The 3rd stage (after 32nd day); Final stage of the degeneration.

The author's experiments have been performed during the stage corresponding to the 2nd one, though the material is not the peripheral but the central nervous tissue. The experiments on degeneration of the optic nerve fibers of the rabbit have been attempted by Suzuki using the rabbit killed from 18 days to 3 weeks after a coagulating puncture of the retina. In this stage, even the optic tract and lateral geniculate body have

been found to be well stained with Marchi's staining; that is, the Marchi stage is already represented. This phenomenon results from degeneration of myelin. It has been said that the degenerating myelin frees neutral fat from phosphosphingoside of myelin or that phosphosphingoside is substituted by non-phosphosphingoside. At any rate, the Marchi stage is considered to coincide with the stage of biochemical disintegration of the myelin sheath.

In the results of the author's experiments in this stage, it is apparently indicated that the prealbumin fraction almost disappears not only in the optic nerve, but also in the white and gray matters. It is difficult to interpret the change of prealbumin in the white and gray matters, but such a disappearance of the prealbumin fraction in the white and gray matters may be influenced by the degeneration which may possibly occurs in the visual pathway upward of the lateral geniculate body and the cortex of the occipital lobe. The detail, however, remains obscure. At any rate, the above-mentioned results may throw some light on the elucidation of property of prealbumin with the consideration of the fact that the prealbumin fraction almost disappears also in the rabbits with allergic encephalomyelitis produced experimentally as described later.

*In the rabbits with allergic encephalomyelitis produced experimentally*:

Recently, the theory of brain tissue allergy has been suggested for the pathogenesis of demyelinating diseases including multiple sclerosis. This theory is considered to be as follows; degeneration of the brain tissue occurs from any unknown cause and the degenerated tissue itself comes to have antigenic property which results in autosensitization. In this way, the antibody is produced and with advance of the consecutive antigen-antibody reaction, the demyelinization proceeds. Freund has succeeded in producing allergic encephalomyelitis experimentally by means of an adjuvant, based upon the fact that antigen for non-specific protein can be produced more easily in tuberculous guinea-pig than in the normal.

The metabolism of lipids is essential for the studies on demyelinating phenomenon as referred to by Edgar. Most of lipids of the nervous tissue, however, exists in the form of lipoprotein or proteolipid. Protein in the myelin sheath especially build up compound substance with lipid, and as for lipids, free cholesterol, cerebroside, sphingomyelin, phosphatidylserin, sulphatid, etc. can be enumerated. As to proteins, neurokeratin closely related to diphosphoinositide is a essential component. Therefore, it is reasonable to believe that some changes in the myelin sheath may result in quantitative changes of protein closely related to lipid.

In the rabbits with allergic encephalomyelitis produced experimentally, disappearance of prealbumin has been demonstrated in the optic nerve, gray and white matters. This result coincides with that of the human experiment by Kitamura and Hara to the effect that the prealbumin fraction shows an outstanding decrease in diseases of the central nervous system such as meningitis, brain tumor, brain trauma and syphilis. A disappearance of prealbumin also has been found to be present in the optic nerve of the rabbits

with Wallerian degeneration as described earlier. Therefore, it is most unlikely that a decrease or disappearance of prealbumin results from a specific change of diseases of the central nervous system.

An increase of the fraction IV has been demonstrated in the optic nerve and white matter. Considering that the fraction IV corresponds to $\gamma$-globulin of the serum, this phenomenon is supposed to originate in the local production of antigen. According to the immunochemical study of Kabat, an attitude of the $\gamma$-globulin fraction of the cerebrospinal fluid in the patient with multiple sclerosis closely resembles that of the cerebrospinal fluid in the monkeys with allergic encephalomyelitis produced experimentally, and a remarkable increase of $\gamma$-globulin of the cerebrospinal fluid has been recognized in 85% of doubtless multiple sclerosis. Furthermore, it has been also reported that only $\gamma$-globulin does not show a decrease in percentage value even after the monkey has completely recovered clinically and the total amount of proteins has become normal again. Such an increase of $\gamma$-globulin in the brain tissue and the cerebrospinal fluid is presumptive evidence in support of allergic theory for pathogenesis of demyelinating diseases. A marked increase of the fraction III has been found in the optic nerve and white matter, but increased amount of fraction IV in the gray matter has been very slight. This is because a main part involving pathologic changes caused by this disease is the white matter.

As for the fraction III, Seki has reported an increase of $\beta$-globuiln corresponding to the fraction III, in addition to an increase of $\gamma$-globulin as referred to by Kabat and others, in paper electrophoretic studies on the proteins in the cerebrospinal fluid of patients with multiple sclerosis. An increase of the $\beta$-globulin fraction is demonstrated in the cerebrospinal fluid of patients with retrobulbar neuritis which gives reise to suspicion of an initial sign of multiple sclerosis, and an increase of the $\gamma$-globulin fraction have not been found. Roboz has reported increased amount of the $\gamma$-globulin fraction in all out of 10 cases and an increase of the $\beta$-globulin fraction in 6 cases. From the above, an increase of the $\beta$-globulin fraction of the cerebrospinal fluid is found in the majority of patients with multiple sclerosis and it is thought that this phenomenon may be closely related to pathochemical findings of disintergration of myelinlipid through demyelinating process. Esser has suggested that an increase of the $\beta$-globulin fraction corresponding to the fraction III, in the crebrospinal fluid results from disintegration of the brain tissue and $\beta$-globulin plays presumably the role of a lipid carrier. The facts that there is an intimate relationship between $\beta$-globulin and lipid, and that in the author's experiments a remarkable decrease of the fraction III is not found in the gray matter seem most likely to support the view that $\beta$-globulin is closely related to a biochemical construction of myelin; lipoprotein, a constituent of myelin, is disintegrated through demyelinating process and a disintegrated lipoprotein may be discharged or absorbed into the blood and cerebrospinal fluid, and then in the brain tissue may give rise to a decrease of $\beta$-globulin; on the other hand, the humor may show its increase. Accordingly, a decrease of the $\beta$-globulin fraction in the optic nerve of the rabbit involving Wallerian degeneration may be

induced by the same mechanism as the above.

From the point of view that protein is a lipid carrier, the investigation on the relation between protein and lipid is scheduled by means of fat staining in addition to protein staining on the filter paper.

By the way, according to a report of Sugawara, even when experimental allergic encephalomyelitis is clinically produced, a close parallel relationship between intensities of clinical symptoms and histopathological changes is not always recognized. In the author's experiment, it has been impossible to confirm histopathological evidence in addition to paper electrophoretic studies using the same experimental animal, because the amount of the material, especially that of the optic nerve tissue is not enough to perform these two kinds of experiments. Therefore, there is no histopathological proof that the optic nerves used for paper electrophoretic experiments have had allergic encephalomyelitis, but compared with experimental results regarding the white matter, it seems justified that the optic nerve might also have been involved.

## Summary

Protein fractions of extracts from the optic nerve tissue of rabbits were investigated by means of paper electrophoresis according to Grassmann and Hanning's method with horizontal paper. This studies were attempted in order to elucidate the pathogenesis of retrobulbar neuritis with special referrence to demyelinating diseases. The so far obtained results are as follows:

1) In the normal rabbits, five fractions were found to be present in both the optic nerve and brain tissues. These protein fractions on the filter paper were designated in order of decreasing mobility as prealbumin, fraction I, II, III, and IV corresponding to albumin, $\alpha$-,$\beta$- and $\gamma$-globulins respectively, except for prealbumin. In both the optic nerve and brain tissue, the fraction I corresponding to the albumin fraction which shows characteristically a sharp high peak in the serum was very low, and the optic nerve tissue exhibited a slack high peak in the fraction III, on the other hand a pattern of the brain tissue showed the slack peaks in the fraction II besides the fraction III.

2) In the rabbits with Wallerian degeneration of the optic nerve produced by removing of both of their eyes, the prealbumin fraction almost disappeared in both the optic nerve and brain tissues. In addition, the fraction III was found to be decreased in the former.

3) In the rabbits with allergic encephalomyelitis produced experimentally, a disappearance of the prealbumin fraction in both the optic nerve and brain tissues, an increase of the fraction IV and a decrease of the fraction III in the optic nerve and white matter of the brain were demonstrated.

An increase of the fraction IV seems to result from antibody formation, and disintegration of the tissue, especially the myelin sheath, caused by demyelinization might be responsible for a decrease of the fraction III.

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# STUDIES ON THE CHEMICAL COMPONENTS OF SCLERA AND CORNEA BY HIGH POTENTIAL PAPER ELECTROPHORESIS.

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## Introduction

High potential paper electrophoresis which was first reported by Michl in 1951 has caught attention as an excellent analytical method for some substances of comparatively small molecular weight. Even in the ophthalmic field, it has been applied to analysis of some tissues of the eye. Miyamoto et al studied its constructions and conditions and discovered the horizontal type electrophoresis from their original approach.

The author and our group, as ophthalmologists, applied it for analysis of some tissues of the eye, namely firstly Yoshizawa observed free amino acid in the fluid of anterior chamber, secondly Takagaki analysed that of vitreous body, and finally Koide that of the lens.

In our department of ophthalmology, Pr. Otsuka has long been investigating myopia, and as a fundamental research of its chemical viewpoint, he commanded me to compare the sclera with the cornea, for these two tissues resemble with each other in origination but differ in development, moreover it is not the cornea, as he indicated, that plays leading role to the genesis of myopia, but the sclera.

For the purpose of comparison, the author analysed the chemical components of the sclera and the cornea under the same conditions in terms of high potential paper electrophoresis and found something of interest among them, so he has reported here about these outlines.

## Methods

*Apparatus of high potential paper electrophoresis.*

The method of Miyamoto et al which was always used in our department was employed in the present investigation.

*Filter paper.*

No. 51 filter paper of Toyo Roshi Co. was cut into 5×40cm size.

*Buffer solution.*

The mixture of acetic acid, formic acid and water (15 : 5 : 80) was used.

*Materials.*

The meterials were extracted from the eyeball of the cattle, pig and horse as soon as slaughtered. After separating, sclera and cornea were separated from the eyeball of these three animals, excepted the corneal limbs, the corneal epithelium and the part of the sclera which belongs to the optic nerve (Foramen fasciculi optici sclerae), then the sclera and the cornea were cut into many small pieces and put into large quantities of methanol for about 24 hours, the methanol extraction was then destilled in the water-bath for 3 hours, put into another glasses, and finally destilled again to dryness. A few drop of water was added in it, then this solution was used for the electrophoretic materials.

*Technique.*

Small amount of sample was applied in linear form at the start line on the paper. High potential direct current was applied through a transformer. As cooler insulation Kerosine was used. In the preliminary experiment, 4 KV, 25 minutes was found to be adequate for these materials. The electrophoresis was first investigated under these conditions. The filter paper which was held up from the pool of the apparatus was dried and stained by 0.2% Ninhydrin aceton solution. The electrophoretic pattern of each sample was observed for its number of fractions, its mobility and the rate of concentration by the aid of densitometer so it was qualitatively and quantitatively investigated. These methods will be described later.

## Results

Number of fractions of each sample was calculated, and through all electrophoretic figures of these samples, was recognized about 11 to 16 fractions.

In these fractions, wider fraction and unusually separated fractions were recognized at the end of the filter paper near the cathode in every pattern, on the otherhand several fractions which were unusually separated and indicated uncertain concentration appeared near the start line. But on the filter paper intermadiate between those two fractions there were almost nine fractions which were usually separated and the concentration were almost always orderly. As above mentioned, there were two types of fractions, namely usually separated fractions and unusually separated ones, which were recorded as a, b, c, and A, B, C,—E etc (those near the cathode were named in small letters, those near the start line were in capital letters), and the former were named Fraction 1 (Fr. 1 etc) through Fr. 9. Among these usually separated fractions Fr. 5 and Fr. 6 approached one another and sometimes appeared as if one fraction. The fraction of which mobility was same as that of Lysine was named Fr. 1 and from this towards to the start line named as Fr. 2—Fr. 9 in that order (Fig. 2).

The mobility of each fraction was calculated as follows:

Fig. 1. Loaded time of high potential paper electrophoresis and its electrophoretic pattern of the cornea of cattle. (from the preliminary experiment).

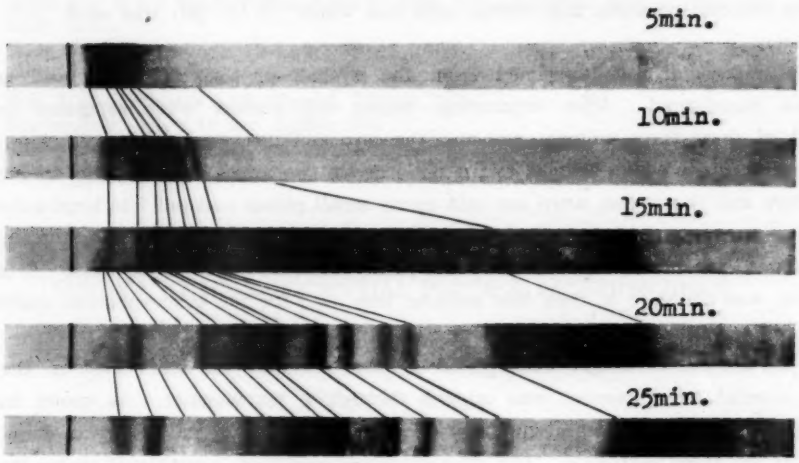
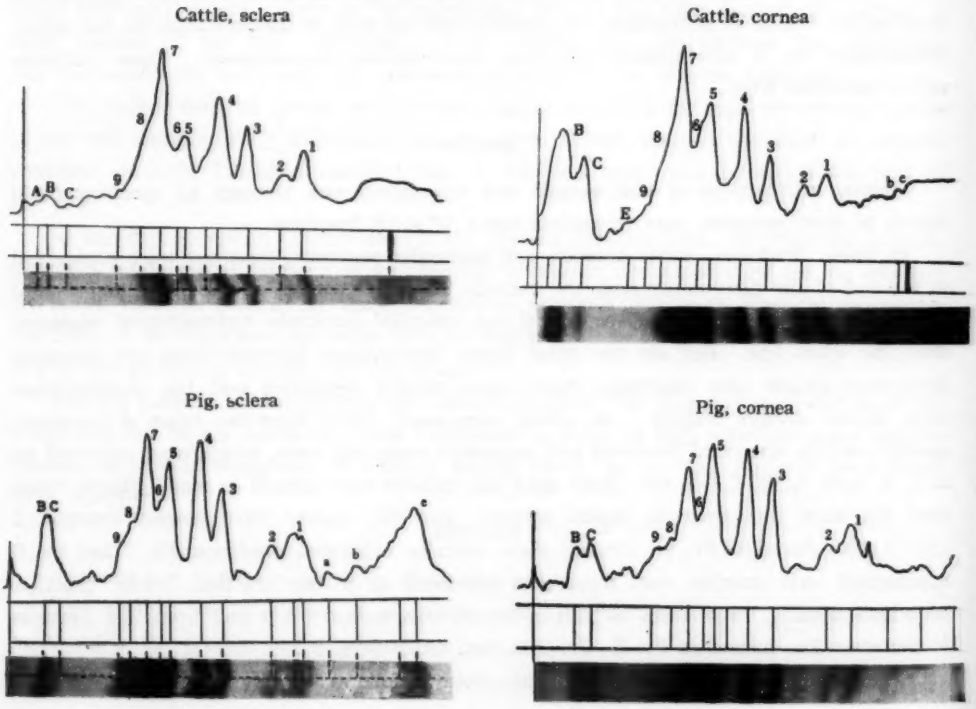



Fig. 2. Electrophoretic patterns of each sample.



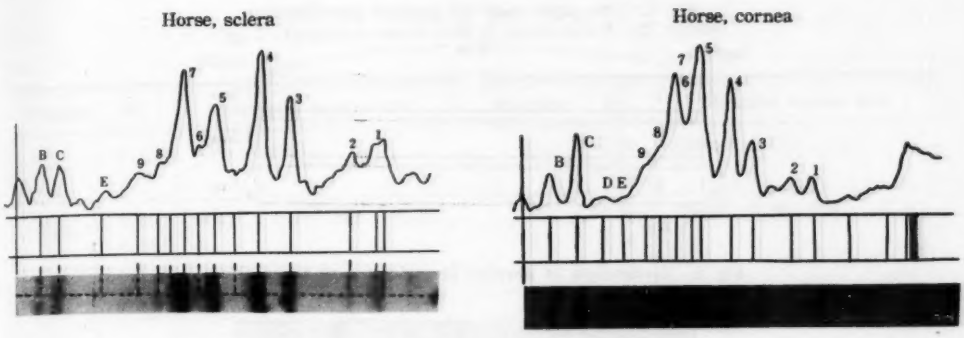
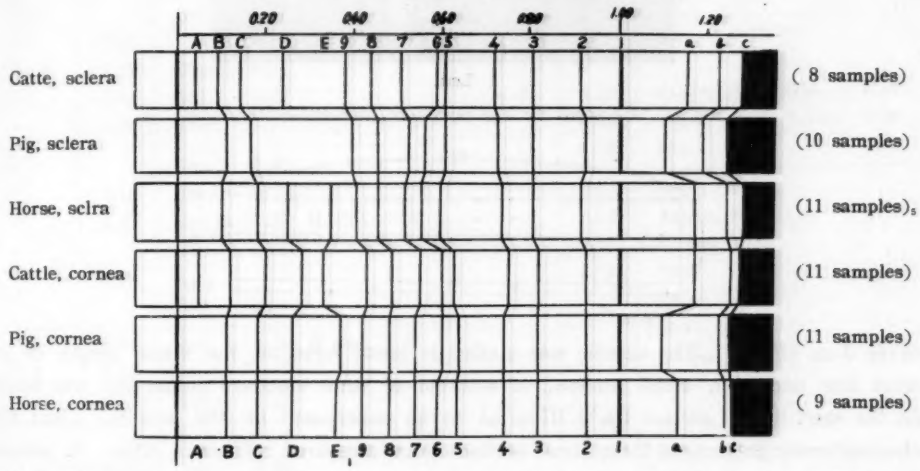



Fig. 3. Mean value of RLys of each sample.




The rate of the distance between start line and each fraction to that of Lysine was named as RLys, and this RLys value of every fractions was indicated for their mean value in Fig. 3. As in Fig. 3 the usually separated fractions, at least, were shown almost same ratio for their corresponded fractions in spite of the different races and different tissues. These facts led the author to believe that in the corresponded fraction there were same or similar substances.

Identification of usually separated fractions of the electrophoretic patterns was next investigated.

The sample was taken from the eyeball of the horse. By means of two methods—so called parallel identification and paperchromatography—using the standard samples of amino acid which was refined by Toyo Kasei Co., the author identified every usually separated fractions. Namely as the method of parallel identification, Toyo filterpaper was cut into 40×10 cm size and the start line was drawn at the distance of 8 cm from its end, and then the paper was devided longitudinaly for three parts and their width were

Fig. 4. The paper used for parallel identification.

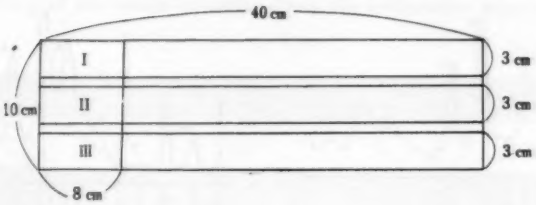



Fig. 5. An example of parallel identification of horse sclera.

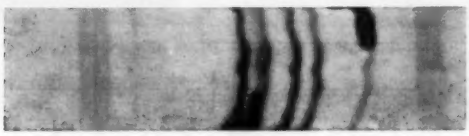
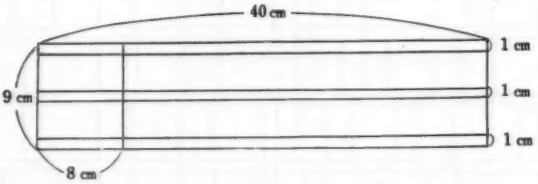



Fig. 6. The paper for the sample of paperchromatography.




taken 3 cm (Fig. 4). The sample was applied in linear form on the whole length of its start line and then small amount of solution of some standard amino acid was added on the start line of section I and III so as to be superposed on the sample. Then the electrophoretic patterns of these three sections were compared with each other. In section I and III, there applied some standard amino acid and sample. On the otherhand, in the section II sample only was applied, so in the electrophoretic patterns of section I and III, some fraction which had the same mobility as that of standard amino acid superposed to the sample on its start line, indicated higher concentration (Fig. 5). By comparing this higher concentrated fraction with its corresponding one, he determined what substances were involved in each fraction. Thus each usually separated fraction was identified by this parallel identification at first, but it was deemed insufficient to decide merely by this method, for there were some amino acid which showed the same mobilities, and was unknown that how many and what amino acid were involved in the corresponding fraction. So the author used the paperchromatography at the same time. As preparatory method of the paperchromatography, filter paper was cut into $9 \times 40$ cm size (Fig. 6). The sample was applied to the start line. After electrophoresis was finished, two edges and the center of this filter paper were cut at 1 cm width by the knife. Only these three sheets of paper were stained by ninhydrin aceton solution and by the aid of locations and

Fig. 7. Identified amino acid of horse sclera and cornea.

Sclera

| Fraction | Rf. | Standard amino acid |
|---|---|---|
| 1 | 0.11 | Lys. 0.11 |
| | 0.18 | |
| 2 | 0.11 | His. 0.09 Arg. 0. 11 |
| | 0.24 | |
| 3 | 0.18 | Gly. 0. 16 |
| | 0.24 | |
| 4 | 0.25 | Ala. 0.27 |
| 5, 6 | | Ser. 0.17 |
| | 0.21 | |
| | 0.23 | Glu. a. 0.24 |
| | 0.52 | Val. 0.53 |
| | 0.70 | Leu. 0.71 I.Leu. 0.82 |
| 7 | | Cys. 0.06 |
| | 0.17 | Ser. 0.17 |
| | 0.21 | Asp. a. 0.20 H. Pro. 0.21 |
| | | Thr. 0.23 |
| | 0.27 | |
| | | Met. 0.52 |
| 8 | 0.20 | Asp. a. 0.20 H. Pro. 0.21 |
| | 0.29 | |
| | 0.33 | Tyr. 0.38 |
| 9 | 0.18 | |
| | 0.23 | Thr. 0.23 |
| | 0.27 | |
| A~E | 0.18 | |
| | 0.24 | |
| | 0.33 | |
| | | Try. 0.52 |
| | | Phe. 0.60 |

Cornea

| Fraction | Rf. | Standard amino acid |
|---|---|---|
| 1 | 0.10 | Lys. 0.10 |
| | 0.15 | |
| 2 | 0.11 | His. 0.10 Arg. 0.11 |
| | 0.14 | |
| | 0.31 | |
| 3 | 0.12 | Gly. 0.13 |
| 4 | 0.13 | |
| | 0.18 | Ala. 0.21 |
| 5, 6 | | Ser. 0.12 |
| | 0.15 | |
| | 0.18 | Glu. a. 0.18 Glu. 0.18 |
| | 0.43 | Val. 0.39 I. Leu. 0.54 |
| | 0.58 | Leu. 0.58 |
| 7 | | Cys. 0.06 |
| | 0.13 | Asp. a. 0.11 |
| | 0.16 | H. Pro. 0.15 |
| | 0.19 | Thr. 0.18 |
| | | Pro. 0.23 |
| | | Met. 0.37 |
| | | I. Leu. 0.54 |
| | | Leu. 0.58 |
| 8 | 0.14 | H. Pro. 0.15 |
| | 0.19 | Thr. 0.18 |
| | 0.53 | Phe. 0.53 |
| 9 | 0.13 | |
| | 0.17 | Asp. 0.17 |
| A~E (10) | 0.12 | |
| | 0.22 | |
| | | Tyr. 0.35 |
| | | Tyr. 0.45 |

forms of these ninhydrin stained fractions on three papers, the positions of fractions on the other nonstained two sheets of papers were presumed and then the portion of the filter paper at presumable positions of fractions were cut by sissors. Small pieces of filter paper thus were cut, were washed by distilled water, condensed, and used as the sample of paperchromatography. Paperchromatography was done using the mixture of n-buthanol, acetic acid and water (4 : 1 : 5) as its solvent and for the primary dimension. Alos Rf values of these fractional samples of paperchromatography were compared with

those of standard amino acid solutions. These two methods enabled the anthor to determine what amino acids were involved in each fraction. Standard amino acid which were used for these two methods for identification were as follows:

Lysine (Lys.), Histidine (His.), Arginine (Arg.), Glycine (Gly.), Alanine (Ala.), Valine (Val.), Leucine (Leu.), Iso-Leucine (I. Leu.), Serine (Ser.), Glutamic acid (Glu. a.), Threonine (Thr.), Methinine (Met.), Phenylalanine (Phe.) ,Cystine (Cys.), Hydroxy proline (H. Pro.), Aspartic acid (Asp. a), Tryptophane (Try.), Tyrosine (Tyr.), Asparagin (Asp.-cornea only).

The results of so-called parallel identification method on the sclera and the cornea of the horse were as follows: Namely Fr. 1 corresponded to Lys.; Fr. 2 to His. and Arg.; Fr. 3 to Gly.; Fr. 4 to Ala.; Fr. 5 to Val., Ser. and I.Leu.; Fr. 6 to Leu. and Thr.; Fr. 7 to Cys., Phe., Glu.a. and Met.; Fr. 8 to Asp. a, H. Pro. and Tyr.; Fr. 9 to Try. No identification was possible on unusually separated fractions.

The results of paperchromatography were complex to some extent, for to determine the substances of the spot on the paper which indicated the same or similar value of Rf to the standard amino acid, he also had simalteniously to refer to the results of previous parallel identification. Thus the chemical components of each fraction of horse sclera and cornea were presumend as in Fig. 7.

From the foregoing results, it was possible to state, at least, more than 11 kinds of amino acid were involued in the samples. However some spots were considered as polypeptide-like substances.

No remarkable differences were recognized between the sclera and the cornea for the mobility and contained substances of each fraction, but characteristic differences of these two tissues were presented on the calculation of concertration. In order to calculate the rate of concentration of each fraction, densitometer and planimeter were employed. Namely on the concentration curves which were drawn by the aid of densitometer, the areas corresponding to each fraction were measured by the planimeter.

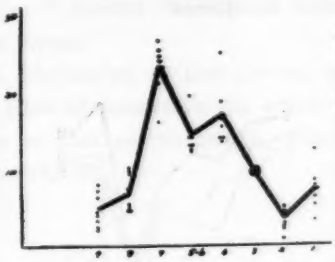
Among these values of areas, as that of Fr. 3 (Gly) indicated comparatively constant value throughout all samples, so its percentage was adopted for the rate of concentration. These were shown in Fig. 8. Fig. 8 was classified by the race of three animals and two tissues. Of the mean value of these Tables, although the race was different, as far as the sclera in concerned, Fr. 7 indicated the highest value, on the other hand Fr. 5-6 was the highest one for the cornea. (Fr. 5 and Fr. 6 appeared as closed fractions, so these were take-up as one fraction as previously mentioned.)

This fact was interesting and most remarkable difference between sclera and cornea from the viewpoint of highpotential paperelectrophoretic patterns, and it was also considered as the characteristic difference of the two.

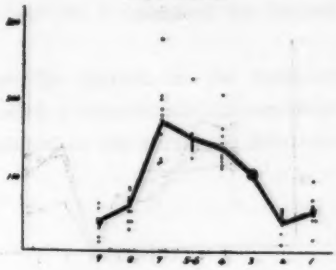
As already mentioned, the rate of concentration of each fraction was expressed by the percentage for Fr. 3 (Gly), but the fluctuation was comparatively large on each fraction through all samples, so the deviation of them had to be considered. These

Fig. 8. The rate of concentration of each sample and its mean value.

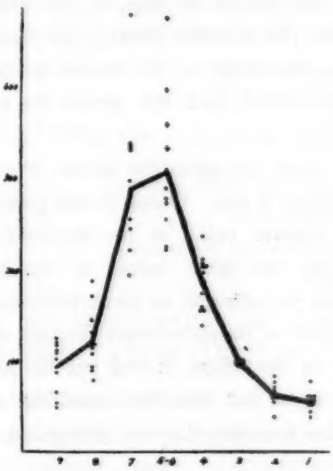
Cattle, sclera



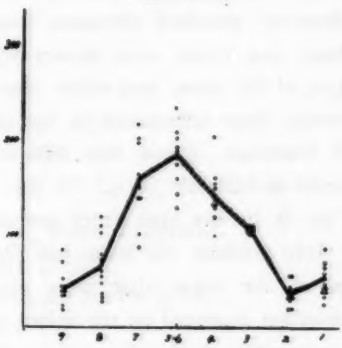
Pig, sclera



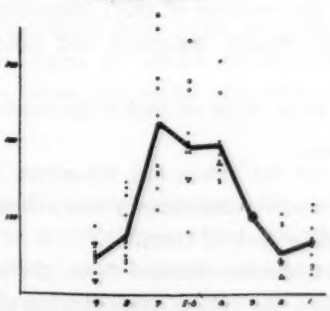
Cattle, cornea



Pig, Cornea



Horse, sclera



Horse, cornea

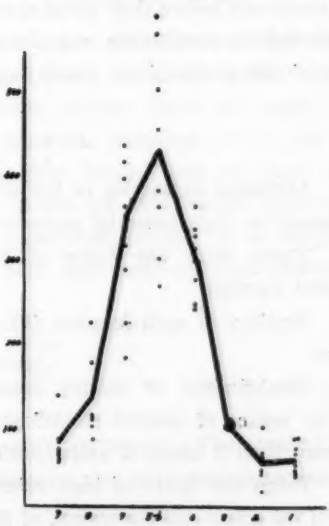
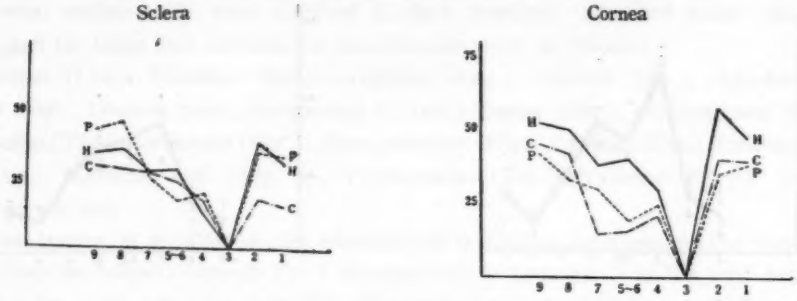


Fig. 9. Standard deviation of sclera and cornea of each animal.




deviations were calculated also by the percentage for the mean values of every fraction.

Moreover standard deviation was calculated in order to consult these characters of deviation, and these were shown Fig. 9. On the sclera, as in Fig. 9, the standard deviation of the horse was wider than that of another two animals through all fractions, conversely, these differences by the race were not so remakable on the cornea except for a few fractions. About this difference between the sclera and the cornea the author presumed as follows:

This is to say that every sample was obtained from the slaughter house, where, of these three animals, the horse had the largest fluctuation of age. Owing to this generative change of the horse that were slaughtered, the highest value of its fluctuations of concentration appeared on the sclera of the horse. On the other hand, on the cornea their generative variations in these three animals has not affected so remarkably as that of the sclera, perhaps because on the cornea, the degree of its metabolism became almost constant already before their slaughtered age, contrally on the sclera, it was yet the process of adult and its metabolism was changeable by the age. But stricktly speaking, determining of this problem, we would require study of the samples of every generation.

## Summary

1. Methanol extraction of the sclera and the cornea of the cattle, pig and horse was investgated by high potential paperelectrophoresis at the condition of 4KV. 25minutes.

2. There were two types of fractions, namely usually separated and unusually separated fraction.

3. Mobility of each fraction (RLys) had almost same value as that-of the corresponding one.

4. Identification of usually separated fractions of the sclera and the cornea of the horse by means of parallel identification method and paperchromatography was investigated and more than ll kinds of amino acids were identified in each of them.

5. From the figure of high potential paperelectrophoresis obtained from the components of the sclera and the cornea of these three animals, the rate of concentration of each

fraction was expressed in percentage for Fr. 3 (Gly). In spite of the difference of the race Fr. 7 showed the highest value on the sclera and Fr. 5-6 showed the highest one on the cornea.

6. Fluctuation of the rate of concentration was the highest on the horse among three kinds of animals on the sclera, but the cornea such a remarkable difference did not appear as that of the sclera. This fact was considered as the metabolic differences of the two tissues.

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## STUDIES ON ALLERGIC REACTIONS CAUSED BY LENS SUBSTANCES

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### II. DETERMINATION OF CARBOXYL-TERMINAL AMINO ACID AND SEDIMENTATION CONSTANT OF ALPHA-CRYSTALLIN.

In the previous study, it was demonstrated on an agar gel diffusion technique that the organ specificity was observed in alpha-crystallin and in one of the beta-fraction bands in several animals' lenses. Alpha-crystallin prepared from bovine, horse and rabbit lenses showed a single and similar pattern on a zone-electrophoresis, revealing nearly the same electrophoretic mobility. On detection of amino-terminal amino acid of these alpha-crystallins, glutamic acid was commonly found. But it has not been decided whether the organ specificity is due to the same structure of alpha-crystallins, or not. It is the purpose of this paper to report carboxyl-terminal amino acid and sedimentation constant of bovine, horse and rabbit alpha-crystallins.

#### Materials and Methods

*Purification of Alpha-crystallin.*

The purification method was described in the previous report. The purified alpha-crystallin showed a single pattern on a zone-electrophoresis and ultra-centrifugation.

*Detection of Carboxyl-terminal Amino Acid.*

S. Akabori's method was used, that is, 30 mg. of alpha-crystallin was treated with anhydrous hydrazin, then was evaporated over conc. $H_2SO_4$ in a desciccator and the dried residue obtained, was dissolved in 10 ml. of water. To this solution enunt aldehyde was added in order to remove hydrazin and hydrazid, and then filtered off. The filtrate was dinitrophenylated with 2-4-dinitro-6-fluoro-benzene for 2 hours, thereafter extracted with ethylacetate at pH 1.0. Carboxyl-terminal amino acids were re-extracted from the extract with 3% solution of $NaHCO_3$ and were identified on two dimensional paperchromatography, using butanol with 2N. $NH_4OH$ for a first run solvent and 1.5 M phosphate buffer for a second run.

*Determination of the Sedimentation Constant.*

Hitachi Model UCA-1 was used. The experimental conditions were as follows: 2% alpha-crystallin solution, in a phosphate buffer (1/40 M. pH 7.6), at 29.5 °C and 32°C.

## Resuls and Discussion

Carboxyl-terminal amino acids of bovine and horse alpha-crystallin were commonly glycine and alanine, but that of rabbit alpha-crystallin was glycine alone. The sedimentation constant of these alpha-crystallins proved as follows;

Bovine alpha-crystallin $S_{29.5}$ : $9.61\times10^{-13}$
Horse alpha-crystallin $S_{32}$ : $15.4\times10^{-13}$
Rabbit alpha-crystallin $S_{32}$ : $17.6\times10^{-13}$

From these results, it becomes obvious, that every alpha-crystallin in these animals is different structurely, so that the organ specificity between these alpha-crystallins may be is due to the similarity of determinant groups in the protein molecules.

## Summary

Determination was made of carboxyl-terminal amino acids and the sedimentation constant of the bovine, horse and rabbit alpha-crystallin. Glycine and alanine were commonly identified on a qualitative analysis of carboxyl-terminal amino acid in cases of bovine and alpha-crystallin, but glycine alone was obtained in a case of a rabbit. Sedimentation horse constant of these alpha-crystallins was individually different. Accordingly, the organ specificity between these animals' alpha-crystallins might be due to the similarity of determinant groups in the protein molecules.

## III. STUDIES ON THE ANTIGENIC STRUCTURE OF LENS PROTEINS.

It has been believed, that the lens proteins play a major role as causative agent of endophthalmitis phacoanaphylactica. It seems that the cataractous lens proteins are denatured or modificated with an unknown mechanism. Since the real mechanism is yet unknown, artificial modifications to lens proteins were employed and their influence on the antigenicity of lens proteins was obsrved. From another point of view, the data obtained will

be useful for the study on the structures of the lens proteins. This paper reports the antigenic structures of lens proteins on an agar gel diffusion technique using bovine lens antiserum and modified lens proteins.

## Materials and Methods

Preparation of Antiserum.

Rabbits were injected intravenously 5 times with 300 mg of bovine total soluble lens proteins in physiologicals aline solution every 3 days, followed by 3 intramuscular injections of the same antigen in Freund's incomplete adjuvant. Two weeks after the last injection, antiserum was obtained by intracardial puncture, then inactivated at 56 °C for 30 minutes and marzonine was added.

Modifications of total soluble bovine lens proteins.

*Blocking of SH radicals was performed by 10 M. parachloromercury benzoate at 25°C for 30 minutes.*

*Methylation of caboxyl radicals.* A hundred miligram of the lyophilized protein was suspended in 10 ml. of cooled methanol and followed by addition of conc. HCL 0.1 ml. and stored for four days at 4 °C. The precipitate was washed with ethylether and dried.

*Acetylation of amino radicals.* One gram of the protein was dissolved in 10 ml. of half saturated $CH_3COONa$ solution, and 0.6 ml. of anhydrous acetic acid was slowly added.

*Modification of the protein with urea and guanidine hydrochloride.* Urea and guanidine hydrochloride was added respectively to one per cent solution of the protein so as to make a final concentration of 6 mol.

*Denaturation with heat.* One per cent solution of the protein was kept for 60 minutes in boiling water bath.

Table 1.

| Antigen | Zone-electrophoretic pattern | Ouchterlony's agar gel diffusion |
|---|---|---|
| Soluble bovine lens protein | $\alpha$-crystallin ($P_1$)<br>$\beta$-fraction ($P_2$, $P_3$) | Alpha-crystallin, which shows organspecificity. Beta-fractions represent 3 lines, ne of which shows organ specificity. |
| Blocking of SH radicals by PCMB | No changes. | No changes. |
| Acetylation | Faster two components precipitated at pH=5.0. | Loss of $\alpha$-crystallins line and the outest $\beta$-fractions line. |
| Methylation | Four componenents were observed, The fastest and the slowest components precipitated at pH=5.0. | No lines developed. |
| Urea treatment | Separation of $\alpha$-crystallin. Accordingly, four components are observed. Two of later components decreased. | No chauges. |
| Guanidine hydrochloride treatment | Separation of $\alpha$-crystallin. Loss of $\beta$-fractions. | Loss of outer two lines of $\beta$-fractions. |
| Treatment by heat | decrease of $\beta$-fractions. | No lines developed. |
| Trypsin digestion | Loss of $\alpha$-crystallin. | Loss of $\alpha$-crystallin line. |

*Trypsin digestion of the protein.* Ten ml. of 2 per cent solution of the protein at pH 7.5 was incubated with 2mg. of trypsin (Merk) for 24 hours at 37°C.

Modification of the purified bovine alpha-crystallin.

Physicochemical and enzymic denaturation was performed on the purified alpha-crystallin followith the above mentioned procedures.

## Results

The results obtained were summarized in the table (Table 1).

## Discussion

Obermeyer, F. and Pick, E.P. (1906) reported, that protein treated with iodine and nitric acid showed serologically a new appearance of specificities to nitro- and iodine-radicals and decreased homologous specificities. Landsteiner, K. pointed out from the experiment on azo-coupled proteins that serologic specificity of the protein was due to the special determinant groups in the protein molecule. T. Hara suggested, that the organ specificity of lens proteins was attributed to the peptide bonds in the proteins. From the above mentioned facts, it is presumed, that the antigenicity of protein molecule is dependent on a special chemical structure. In this paper, the antigenicity of the proteins was examined with an agar gel diffusion technique, using bovine lens antiserum and modified bovine lens proteins.

There have been many studies, which show the lens proteins are rich in SH radicals. But after the treatment with PCMB, nitroprusside reaction became completely negative, and yet the treated protein reacted with the antiserum. Accordingly, it may be safely said that SH radicals in the proteins have no relationship to antigenicity. In a case in which the purified bovine alpha-crystallin was used, the same results were obtained.

The acetylation of free amino radicals in the protein with anhydrous acetic acid is commonly accepted to be specific. By this procedure, a didappearance of the alpha-crystallin line, and one of the beta fraction lines on agar diffusion method was observed. It is suggested, therefore, that the antigenicity represented by these lines had relation with free amino groups. On the other hand, no precipitation lines between anti-lens serum and protein treated with methanol and hydrochloric acid were observed. But there still remains a question, whether the action of these reagents was specific to the carboxyl groups or not.

It is generally believed, that urea and guanidine hydrochloride cut off the hydrogen bonds in protein molecule, unfolding the peptide chains and increasing the reactivity of concealed radicals. Kinoshita, J.H. reported that an increased extractability of combined glutathiones, and an increased reductability of SH radicals, were demonstrated after lens proteins were treated with urea and guanidine hydrochloride. On agar plates, no changes were observed following the treatment of urea, and two of the beta fraction

lines were extinguished following the treatment of guanidine hydrochloride. This may suggest the difference in modes of actions between the two reagents, i.e. the superficial structure of the molecule, which may have relations with antigenicity, might remain unchanged by urea, while guanidine hydrochloride might cause not only an inner unfolding of the peptide chains, but partial destruction of the superficial structure.

None of the precipitation lines developed on agar plate by thermal denaturation. This indicates, that the antigenicity of the lens proteins represented by these lines shows heat-labile property.

After a 24 hour incubation with trypsin, the alpha-crystallin disappeared completely on a zone-electrophresis but beta-fractions still remained. The precipitation line corresponding to the alpha-crystallin did not develop. Furthermore, the enzymic digestion products of the purified alpha-crystallin did not inhibit the development of the precipitation line between purified alpha-crystallin and its antiserum. Accordingly, it was presumed that the antigenicity of the alpha-crystallin was due to the chemical structure which was destroyed by trypsine, most probably lysine or arginine peptide.

It is generally accepted that the molecular weight of the bovine alpha-crystallin is nearly a million and that of the beta-fraction is several hundred thousand. A qualitative analysis of amino acids composition was performed by Francois, J. et al. on alpha-crystallin, and by Y. Mimura on purified alpha-crystallin. Resnik determined the sedimentation constant and the diffusion constant of the bovine cortical alpha-crystallin and calculated its molecular weight. But it must be admitted, that further details in the structure of the lens proteins are still left unclarified. Further studies seem to be necessary on purified proteins with solubility test, light scattering method, viscosity test and analysis of arrangements of peptide chains, together with studies on purification of other lens proteins.

## Summary

Studies were made of the antigenicity of soluble lens proteins from a view of the chemical structure with an agar gel diffusion method, using bovine lens antiserum and modified lens proteins. The results obtained were as follows;

1. No changes were observed in the antigenicity of bovine-lens-proteins after SH-groups were blocked with PCMB. Thus, the antigenicity of the protein did not seem due to SH-groups.

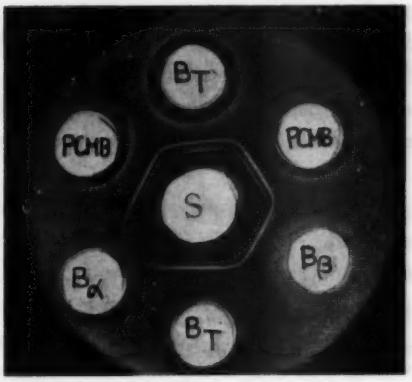
2. On thermal denaturation of the protein and methylation of free carboxyl groups, no antigenicity of the protein was demonstrated.

3. The alpha-crystallin was stable, when treated with urea and guanidine hydrochloride. It was labile to denaturation by heat and destroyed by incubation with trypsin and selective acetylation.

4. One of the three precipitation lines, corresponding to the beta-fractions, was readily destroyed by denaturating agents.

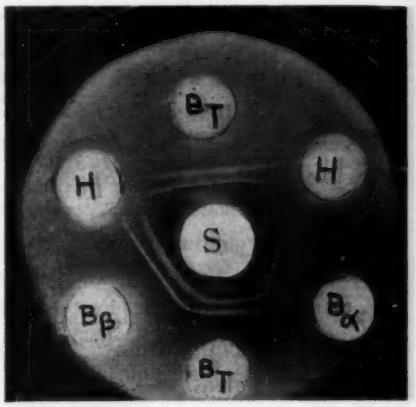
Fig. 1. Ouchterlony's Agar Gel Diffusion Method

a) Blocking by PCMB



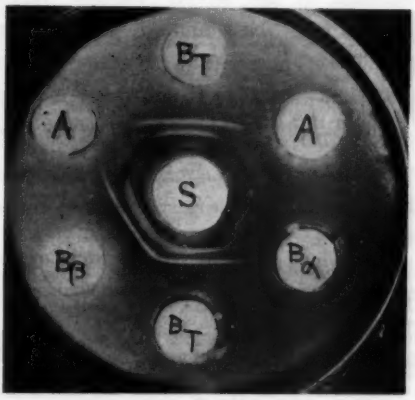

S

c) Heat Denaturation




S

b) Acetylation

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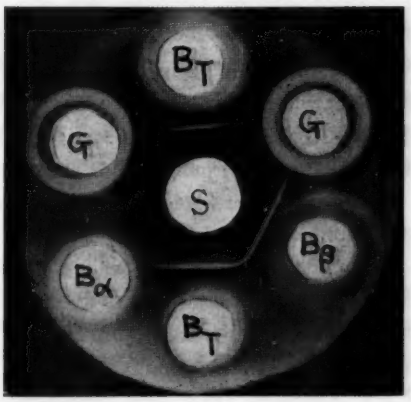
b) Denaturation with Guanidine Hydrochloride




S

S: antisera

Bα: bovine α-crystallin

Bβ: bovine β-fractions

PCMB: Lens proteins treated with PCMB

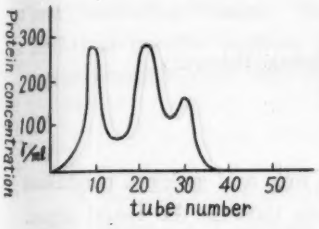
A: acetylated lens Probein

H: Lens proteins denatured by heat

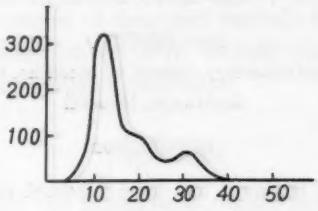
G: Lens proteins denatured with guanidine hydrochloride

Fig. 2. Electrophoretic Pattern

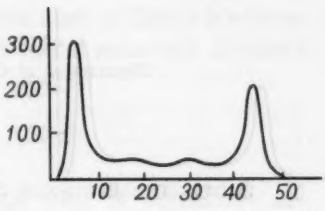
a) Normal Bovine Lens Protein



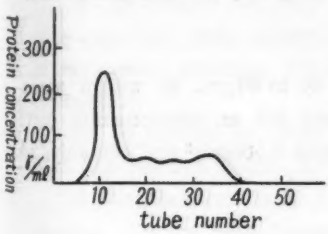

b) Acetylation



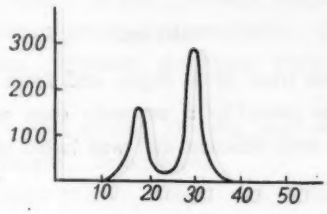

c) Methylation



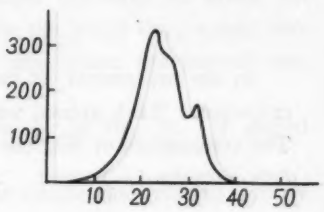

d) Denaturation with Unea



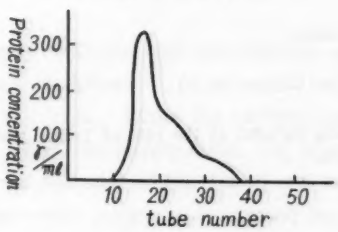

e) Denaturation by Guanidine Hydrochloride




f) Heat Denaturation




g) Trypsine Digestion

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# EXPERIMENTAL STUDIES ON OCULAR MANIFESTATIONS OF FOLIC ACID DEFICIENCY

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## Introduction

A considerable amount of research has been reported on folic acid deficient conditions in a variety of animals, but little work has been done to study those in the visual organ. The present study was designed to make rats show folic acid deficiency and observe the changes in the visual organ of the animals clinically, histologically and histochemically.

## Method

In the experiment, 60 rats from 20 to 40 gm. and from 60 to 80 gm. in weight were employed. Each animal was placed in a separate cage and fed an experimental diet. The composition of the folic acid deficient diet was based upon György's[2] and Ninomiya's[4] diets as given in Table 1.

Table 1.

| | |
|---|---|
| Basal diet | |
| Sucrose | 69.0% |
| Vitamin free casein | 20.0 |
| Cotton seed oil | 6.7 |
| Salt mixture (Mc Collum No. 5) | 4.0 |
| Methionin | 0.3 |
| Sulfathiazole was included at the rate of 1.0gm. per 100gm. of diet. | |
| Vitamin supplements | |
| Inositol | 10.0mg. |
| Vitamin $B_1$ | 0.5 |
| Riboflavin | 0.8 |
| Pyridoxine hydrochloride | 0.5 |
| Nicotinic acid | 4.0 |
| Calcium pantothenate | 4.0 |
| Biotin | 0.04 |
| Vitamin $B_{12}$ | 0.003 |
| Choline | 100.0. |
| Vitamin K | 0.5 |
| Vitamin E | 3.2 |
| Vitamin A | 5000i.u. |
| Vitamin D | 1500i.u. |

For the control animals, 0.2 mg. of folic acid were provided.

The folic acid deficient and control animals were observed clinically, hematologically and by a microbiological assay for the urinary excretions of folic acid during the breeding period. Pieces of organs from dead or decapitated animals were assigned for histological and histochemical examinations. Whole blood, liver and kidney were used for microbiolog-

ical assay. The value of folic acid was determined with streptococcus faecalis "R" ATCC 8043. Specimens of organs were stained with hematoxylin-eosin, azan, Sudan III and Berlin-blue stains. Specimens of liver and eyeballs were fixed in Carnoy's solution and their paraffin sections were stained with periodic acid Schiff stain and Brachet's pyronin-methylgreen stain and Feulgen's technique.

## Experiment

In this report are described the exprimental results gained from 16 satisfactory animals among the 60 animals which were studied.

Control animals.

No. 28 Female rat, body weight 16 gm. Growth continued normally till about the 30th day, the body weight being constant subsequently. On the 100th day, weight was gained again, reaching 108 mg. Physical conditions were good when the animal was decapitated.

No. 30 Female rat, body weight 65 gm. Growth was similar to No. 28. It gained flesh and reached 202gm. in weight. Decapitated.

No. 54 Male rat, body weight 32 gm. Weight was gained continuously during control feeding. The rat was decapitated on the 62th day.

No. 72 Male rat, body weight 62 gm. It grew well and was decapitated on the 45th day.

These control animals were clinically and histologically normal. Specimens from them revealed histochemical findings as follows:

*Periodic Acid Schiff's stain (PAS).* While the external and middle layers of the corneal epithelium were stained in light red-purple color, the internal layer was stained a deep red-purple color. The external segment of the visual cell layer was stained a deep red-purple color in the retina, but other layers were stained only slightly.

*Pyronin-Methylgreen stain (P-MG).* The nucleus was stained in green or purple, and cytoplasma showed in granular form, a positive reaction upon the pyronin in the epithelium of the cornea. Although pyroninphile granules were few in number and located at both sides of the nucleus in the external layer of the corneal epithelium, these became greater in number and wider in distribution in the inner layer. Some nuclei included pyroninphile. The internal segment of the visual cell layer in the retina was stained a deep red color and other layers were stained only lightly. The retinal nucleus was stained in deep green or purple color in the external molecular layer, but only lightly in the internal molecular and ganglion cell layers.

*Feulgen's reaction.* The epithelial nucleus of the cornea was slender in a traverse direction and more round in the internal layer. The epithelial nucleus of the cornea was round, stained to an equal depth in red-purple color with only a slight stain in the external layer. The retinal nucleus was stained in the pigment epithelial layer, and only

slightly stained in the internal molecular and ganglion cell layers.

Deficient animals.

No. 6 Female rat,body weight 75 gm. Grew well to the 15 th day, but growth stopped afterwards. Although the animal grew again from the 30 th day and weight increased to 148 gm. on the 50th day, its growth stopped or rather was disposed to decline subsequently. It lost in flesh, but kept the gloss of fur. Conspicuous physical changes were not observed. On reaching the 100 th day, filthiness of fur, bloody crust of ears, pallor of the retina, dilatation of retinal veins began to be observed. It was decapitated on the 145 th day.

Specimens of eyeball revealed slight deformation of corneal epithelial nucleus, and small edema and hemorrhage in the retina.

No. 7 Female rat, body weight 70 gm. Grew to the 40th day, whereafter growth stopped. It had a good appetite and gloss of fur. While the bloody crust on the right eye-lid and nose were observed on the 100 th day, neither non-instrumental nor ophthalmoscopic changes were observed in the eyeball. It was decapitated.

Specimens revealed slight deformation of corneal epithelial nucleus and stasis of retinal blood vessels.

No. 15 Male rat, body weight 70 gm. Growth stopped on the 60 th day and the fur of head, nose and abdomen became a dirty brown on the 100 th day. It lost flesh, but was relatively active in motion. There were no ophthalmoscopic changes observed. From the 170 th day body weight inclined to decrease, being 135 gm. on the 180 th day, when the animal was decapiteted.

The epithelium of the cornea was thinner than that of the control animals.

No. 16 Female rat, body weight 78 gm. Growth was slow but constant till the animal reached a weight of 175 gm. on the 15 th day. Subsequently the growth stopped and ophthalmoscopically dilatation and tortuosity of retinal veins were observed. From about the 200 th day on, it became emaciated which culminated in death.

Histological specimens revealed the dilatation of the choroidal blood vessel and stasis of blood vessels in the iris and retina.

Immediately after the death histochemical estimation was done. The PAS-stain colored the middle layer in light red-purple and the internal layer in red in the cornea. Specimens were generally stained somewhat lightly as compared with the control. Although the ganglion cell layer was not stained in the retina, other layers were stained as well as those of the control animals. In the cornea P-Mg-stain colored only the surrounding of the nucleus in red, and the granules in the cytoplasma were scattered and stained more lightly. The nucleus was stained bluish green. While other layers responded to pyronin in the same order of depth as the control, the ganglion cell layer responded only faintly. By Feulgen's technique, the epithelial nucleus of the cornea was almost normal. The nucleus of the ganglion cell layer was lightly stained in the retina.

No. 21 Male rat, body weight 65 gm. Growth stopped on the 40 th day. The animal

had a good appetite, was active but lost flesh. Dilatation of the retinal veins was observed ophthalmoscopically. From about the 180 th day, the loss of body weight began to cause loss of gloss to the fur. The animal died on the 203 rd day.

Histological specimens revealed the deformation (pyknosis) of the corneal epithelial nucleus.

PAS-stain showed light red color in the epithelium of the cornea. By P-Mg-stain, the cytoplasma was stained equally in light red color, while the deep reddish pyroninphile granule was not seen in the epithelium of the cornea. The nucleus was stained a green color. Feulgen's reaction revealed irregular staining of the nuclear membrane and light staining or partially deep staining of the nuclear substance in the middle and internal layers of the corneal epithelium.

No. 22 Female rat, body weight 60 gm. grew to the 50 th day. There was no subsequent growth. It had a good appetite and was relatively active. From the 90 th day, flesh was lost, and weight decreased. The nose was filthy and a dilatation of retinal veins was observed. It was decapitated as it became to emaciated.

Histological specimens revealed the deformation (swelling) of some corneal epithelial nucleus, but conspicuous changes were not seen.

PAS-stain showed, that the corneal epithelium stained a light red color. The retina was almost normal. The epithelium of the cornea included red pyroninphile granules, as observed in those of the control animals. The retina was also stained into the same depth as the control. By Feulgen's technique, the nucleus of the middle and internal layers of the corneal epithelium was stained lightly and unequally. There were no specific descriptions in the retina.

No. 24 Female rat, body weight 60 gm. Growth stopped on the 40 th day and body weight began to decrease gradually. On the 90 th day much flesh was lost and it became inactive. Furthermore, body weight decreased and the hair-condition of the fur had a shaggy appearance. The retina was somewhat pale. The animal was decapitated.

Specimens revealed the dilatation of blood vessels, small edema and maldisposition of ganglion cells in the retina.

No. 26 Male rat, body weight 63 gm. Grew well to the 60 th day. Subsequently sometimes weight was gained, sometimes decreased. Gross changes in the eyes and body were not observed.

Specimens revealed swelling of the corneal epithelial nucleus.

No. 51 Male rat, body weight 30 gm. Grew to the 40th day and then growth stopped. From the 66th day, body weight began to decrease, and a bloody crust on the ears, nose and eye-lids was observed. Dilatation of the retinal veins was observed ophthalmoscopically. On the 80 th day the fur of the back fell out and the anime became too emaciated, and was decapitated.

Histological specimens revealed pyknosis of the nucleus in the middle and internal layers of the corneal epithelium. The dilatation and stains of retinal blood vessels were

observed.

By PAS-stain, the epithelium of the cornea was made a purple color. In the retina, the molecular layer, external segments of the visual cell layer were stained a red-purple color, and the plexiform layer was stained a light red-purple color. These colors were lighter in comparison to the control colors. The nerve fiber layer showed hardly any stain. By P-Mg-stain the color of the corneal epithelium was made generally light red and even the internal layer, rich in pyroninphile granules was stained a red color only around the nucleus. The nucleus was stained a green-blue-color. The ganglion cell layer was stained lightly in the retina, but other layers more deeply, as seen in the control retinas. By Feulgen's technique, the epithelial nucleus of the cornea was stained in a mixture of deep and light red colors, accordingly chromatins were arranged irregularly. The ganglion cell layer was a lighter red-purple, but other layers were stained normally in the retina.

No. 37 Male rat, body weight 20 gm. Grew well to the 30 th day when growth stopped. Body weight was 98 gm. on the 120 th day. It has remarkably lost in flesh and became inactive in motion. Gross changes in the visual organ were not observed. On the 8 th day after the subcutaneus administration of folic acid (1 mg) a little increase of body weight and activity was observed. However it continued to lose flesh as before.

Pathological changes were not revealed microscopically in the visual organ.

No. 45 Male rat, body weight 30 gm. Weight was 95 gm. on the 115 th day. Considerable flesh had been lost and a bloody crust on the eye-lids and ears was observed. Dilatation of the retinal veins was recognized ophthalmoscopically. Growth could not be resumed by the administration of folic acid. Gross physical conditions were also not improved. Histological specimens did not reveal any change from that observed in other deficient animals.

## Results

The results of this experiment are summarized as follows:

At first the growth rates of the deficient and control animals were identical from 30 to 40 days in animals of 30 gm. of body weight, and from 50 to 60 days in animals of 70 gm. of body weight. From these stages the growth of deficient animals began to decline and lose of flesh occurred. Some of the deficient animals lost weight and were emaciated. Deficient animals which neither gained nor lost weight retained their gloss of fur and were relatively active. The fur of deficient animals altered a filthy brown on about the 100 th day, and the animal's fur became dull and shaggy in appearance, as they became emaciated. However, their appetite was relatively good. Fall out of fur was also observed in deficient animals. Formation of a bloody crust was seen on the eye-lids, ears and noses of deficient animals on the 100 th day of the experimental diet, but massive bleeding was not recognized in any deficient animal.

Hematological results are shown in Table 2. Examination of hemoglobin disclosed a

Table 2. Hematologic Changes in Various Samples

| | Deficient rats | | | | | | | | Control rats | |
|---|---|---|---|---|---|---|---|---|---|---|
| Number of the rats | 6 | 7 | 15 | 22 | 24 | 26 | 48 | 51 | 28 | 54 |
| Days of diet | 145 | 180 | 180 | 204 | 139 | 140 | 90 | 87 | 139 | 63 |
| Hemoglobin (%) | 87 | 85 | 90 | 78 | 70 | 82 | 90 | 80 | 87 | 95 |
| Erythrocytes millions /cmm | 628 | 640 | 585 | 600 | 550 | 650 | 680 | 580 | 700 | 740 |
| Leucocytes per cmm | 6400 | 5400 | 5200 | 5800 | 4800 | 6000 | 8000 | 5000 | 11400 | 11000 |
| Thrombocytes thousands /cmm | 403.5 | 425.6 | 380.0 | 385.0 | 463.0 | 500.0 | 534.0 | 362.4 | 558.0 | 584.8 |

Table 3. Ratio of Leucocytes in Detail

| | Deficient rats | | | | | | | | Control rats | |
|---|---|---|---|---|---|---|---|---|---|---|
| Number of the rats | 6 | 7 | 15 | 22 | 24 | 26 | 48 | 51 | 28 | 54 |
| Days of diet | 145 | 180 | 180 | 204 | 139 | 140 | 90 | 87 | 139 | 63 |
| Neutrophils (%) | 21 | 14 | 12 | 9 | 16 | 21 | 28 | 17 | 33 | 43 |
| Lymphocytes (%) | 75 | 78 | 82 | 81 | 78 | 69 | 68 | 77 | 56 | 51 |
| Eosinophils (%) | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 2 | 1 |
| Monocytes (%) | 3 | 8 | 4 | 8 | 6 | 9 | 5 | 4 | 9 | 5 |

Table 4. Value of Folic Acid in Various Samples

| | Deficient rats | | | | | | | Control rats | |
|---|---|---|---|---|---|---|---|---|---|
| Number of the rats | 6 | 7 | 15 | 22 | 24 | 26 | 51 | 28 | 54 |
| Days of diet | 145 | 180 | 180 | 204 | 139 | 140 | 87 | 139 | 63 |
| Whole blood mμ/cc | 4.5 | 6.6 | 7.7 | — | 7.6 | 9.6 | — | 18.2 | 10.5 |
| Urine mγ/day | 3.0 | 3.8 | — | — | 3.1 | 7.8 | — | 100 | — |
| Liver mγ/ | 144 | 525 | 720 | 660 | 150 | 450 | 660 | 1080 | 1620 |
| Kidney mγ/gm | 150 | 450 | 528 | 420 | 288 | 340 | 420 | 1080 | 1440 |

little lower percentage in deficient animals. Red blood cells and thrombocytes were reduced to some degree. A noticeable hematological change was leucopenia, and its differential count showed reduced neutrophil leukocytes (Table 3). Folic acid deficient conditions of the experimental animals were elucidated by a microbiological determination of folic acid concentration in the blood, urine, liver and kidney (Table 4). Urinary excretion of folic acid in deficient animals was remarkably decreased. The concentration of folic acid in the whole blood, liver and kidney was also decreased.

Results of histological examination of other organs excluding the visual organ revealed a cloudy swelling, peripheral or diffuse fatty infiltrations in the liver cells, hemosiderin-deposition in the spleen, edema and urinary calculus in the kidney, small hemorrhages in the heart, and cloudy swelling in the muscle fiber of the heart. Histochem-

ical estimation revealed the decrease of glycogen and ribonucleic acid contents in the liver. Feulgen's reaction of chromatin in the nucleus was irregular.

Gross changes in relation to the visual organ did not appear. although the above mentioned blood crust was seen on the eye-lids. Ophthalmoscopic examination revealed congested retinal veins on the 100 th day, and some fundi of deficient animals appeared somewhat pale. However retinal hemorrhage and white patch were not found. Specimens of cornea revealed microscopically deformation of the corneal epithelial nucleus, especially the nucleus in the middle and internal layers. The corneal epithelial nucleus of deficient animals revealed pyknosis and swelling. In one of the specimens the thickness of the corneal epithelium was thin compared with those in the control animals. Such changes of the corneal epithelium appeared somewhat preeminent in No. 21 and NO. 51 or the deficient animals. In the retina, slight edema and small hemorrhages were microscopically found. Some of the specimens revealed dilatation and stasis of blood vessels in the retina, choroid and iris. Histochemical estimation was made on the cornea and retina of four deficient rats. The content of glycogey was commonly less than normal in the corneal epithelium and in some of the retinas. The content of ribonucleic acid was more or less below normal in the corneal epithelium with the exception of No. 22. Estimation of ribo-nucleic acid content in the retina showed its decrease in the ganglion cell layer, but a normal content in the other layers. The content of desoxyribonucleic acid in the corneal epithelium tended to be below normal. Microscopic examination revealed a relatively preeminent deformation of the corneal epithelial nucleus. Chromatin was contained in unequally density in the nucleus. The content of desoxyribonucleic acid in the retina was nearly indentical with that of the controls or was slightly less in the ganglion cell layer of some of deficient animals.

## Discussion

The generalized symptoms of folic acid deficiency in animals which have been reported by many investigators are disturbance of growth, absence of appetite, emaciation, filthiness, shedding of fur and diarrhea. The chief features of folic acid deficiency observed in the present experiment were disturbance of growth, loss of flesh, emaciation, filthiness, and shedding of fur in some animals. However in general, the progress of deficiency symptoms appeared to be relatively slow and chronic. The characteristic hematological picture was leucopenia. As it has been mentioned, the rats were attacked by leucopenia or agranulocytopenia and chick macrocytic anemia.

Histological examination in other organs excluding the visual organ demonstrated cloudy swelling and fatty infiltration in liver cells, hemosiderin-deposits in the spleen, edema and urinary calculus in the kidney, cloudy swelling in the muscle fiber of the heart and small hemorrhages in the heart. The contents of glycogen, ribonucleic acid and desoxy-ribonucleic acid were shown to be less than normal by histochemical examination in the liver. Although these changes coincide with those reported by Miller[3], Gross[1], Reid[5] and

Weir[7], such changes as necrosis were not noticed at all by them.

Finally regarding the visual organ in folic acid deficient animals, the thinness of the cornea, deformation of corneal epithelial nucleus, slight edema and small hemorrhage in the retina, and dialtation and stasis of blood vessels in the retina, choroid and iris were observed. Furthermore it was proved through histochemical examination that the amount of glycogen decreased in the epithelium of the cornea and in some retinas. The amount of ribonucleic acid decreased slightly in the epithelium of the cornea and in the ganglion cell layer of the retina, and the amount of desoxyribonucleic acid decreased in some corneal epitheliums and decreased slightly in the ganglion cell layer of the retina.

Only a few reports of details of the visual organ of folic acid deficient animals have been made. In 1958 Takahashi[6] in our Ophthalmological Department, performed a Vitamin $B_{12}$ deficiency experiment using rats, which proved disorder in all layers of their retina, hypertrophy of the choroid, and dilatation and stasis of choroidal blood vessels. In the present folic acid deficiency experiment, using rats, the author would emphasize that changes in the epithelium of the cornea were more frequently found than changes in the retina. The described changes in the visual organ were reversible changes which were possible to be recovered easily by the administration of folic acid. Folic acid deficiency symptoms are characteristic in a hematological phase as well as in vitamin $B_{12}$ deficiency symptoms, namely macrocytic anemia or leucopenia. Therefore it is not too difficult to presume, that the retina of folic acid deficient animals is affected by these hemotological changes. But although the author observed changes in the epithelium of the cornea, these were unexpected changes. A connection between folic acid and the visual organ derived from changes in the visual organ could not be ascertained. Furthermore much research should be done in future.

## Summary

Rats in weight from 20 to 80 gm. were employed in the experiments and fed on György and Ninomiya's folic acid deficient diets, resulting in the course of the observation feeding from 3 to 6 months.

1. Changes in the visual organ:

Clinically formation of bloody crust was recognized on the eye-lids, and ophthalmoscopic examination revealed congested retinal veins and sometimes pale fundi.

As histological findings mostly deformation of corneal epithelial nucleus and rare congestion, edematous swelling and small hemorrhage of the retina, also dilatation of blood vessels in the choroid and iris were observed.

With histochemical techniques a decrease of glycogen in the corneal epithelium and retina, also a decrease of RNA and DNA in the corneal epithelium and sometimes in the ganglion cell layer of the retina were found.

2. Changes in other organs:

Turbid swelling and fatty infiltration in the liver cells, hemosiderin-deposition in the

Fig. 1. Fatty infiltration in liver cell.
(Sudan III, ×400)

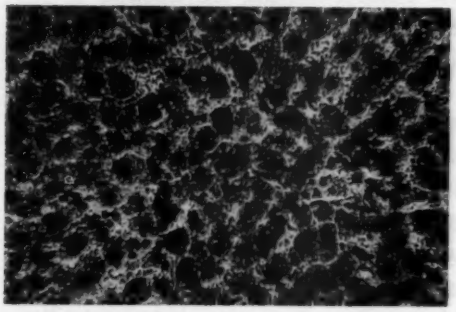


Fig. 2. Hemosiderin in spleen.
(Berlin-blue, ×300)

Fig. 3. Deformation (piknosis) in epithelial nucleus of cornea.
(Hematoxylin-eosin, ×1350)

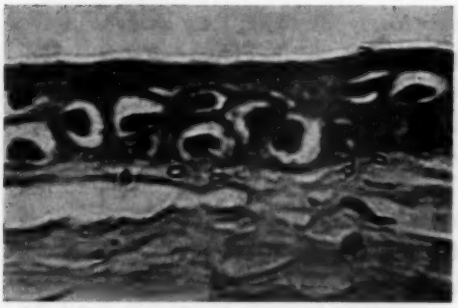


Fig. 4. Deformation (swelling) in epithelial nucleus of cornea.
(Hematoxylin-eosin, ×1350)

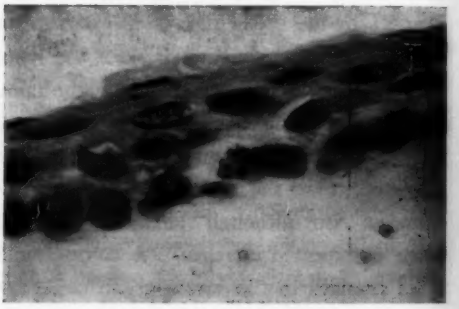


Fig. 5. Edema and small hemorrhage in retina.
(Hematoxylin-eosin, ×900)

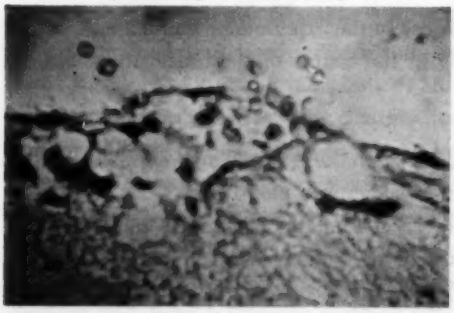


Fig. 6. Dilatation of choroidal blood vessels.
(Hematoxylin-eosin, ×990)

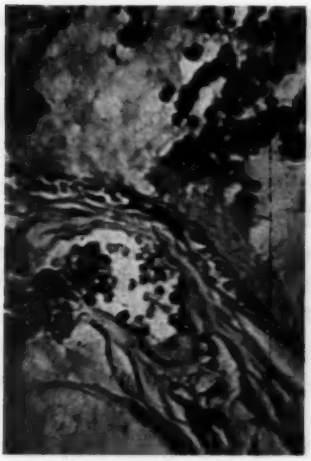


Fig. 7. Liver of control rat. (Feulgen, ×1350)

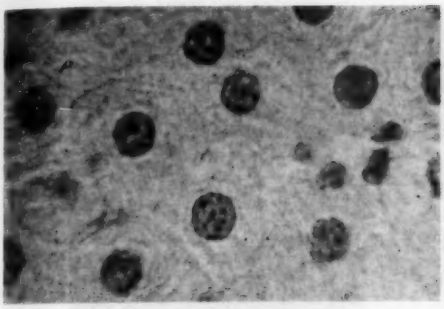


Fig. 8. Liver of deficient rat. (Feulgen, ×1350) Reactive nuclear membrane is unequally and chromatin in the nucleus is decreased.

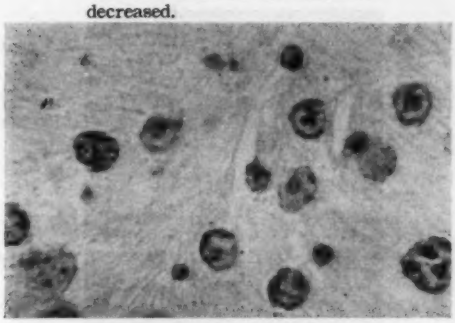


Fig. 9. Corneal epithelium of control rat. (P-MG, ×1350)

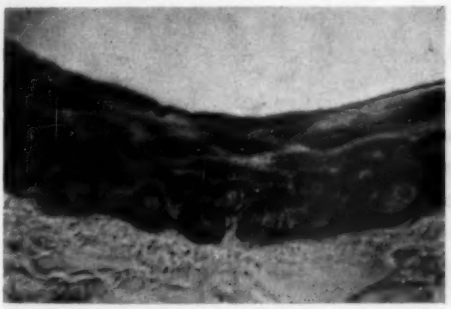


Fig. 10. Corneal epithelium of deficient rat. (P-MG, ×1350)

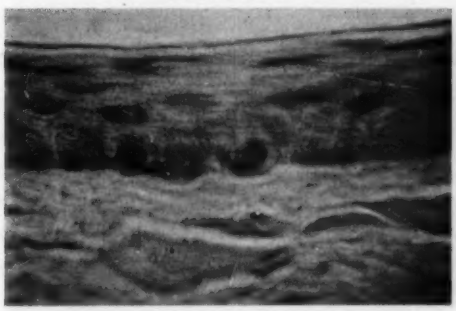


Fig. 11. Content of ribonucleic acid is decreased. Corneal epithelium of control rat. (Feulgen, ×1350)

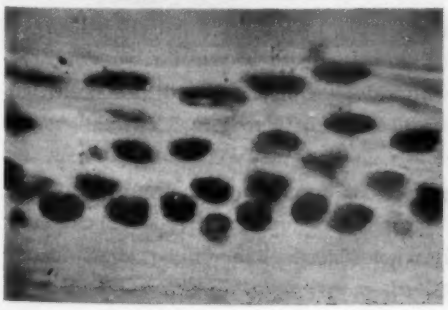


Fig. 12. Corneal epithelium of deficient rat. (Feulgen, ×1350) Content of desoxyribonucleic acid is decreased.

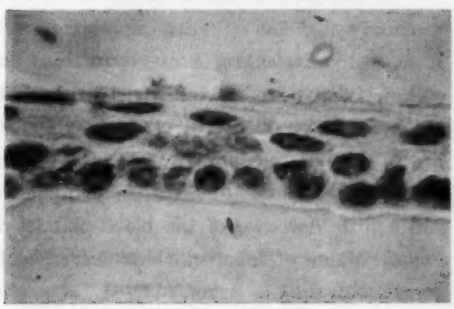


Fig. 13. Retina of control rat.
(P-MG, ×1350)

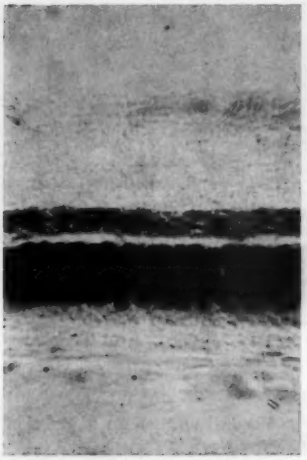


Fig. 14. Retina of deficient rat. (P-MG, ×1350)
Content of ribonuclic acid is slightly in ganglion cell layer of retina.

Fig. 15. Retina of control rat.
(Feulgen, ×1350)

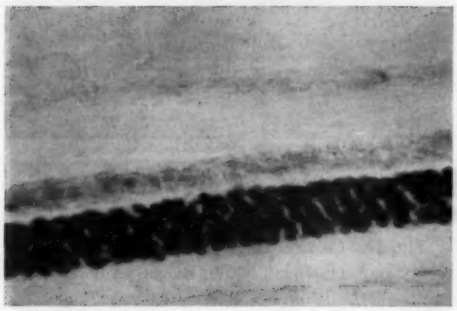
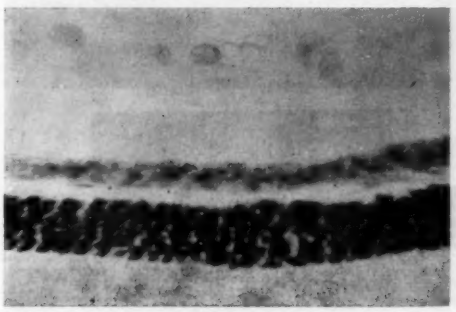


Fig. 16. Retina of deficient rat. (Feulgen, ×1350)
Content of desoxyribonucleic acid is slightly decreased in ganglion cell layer of retina.



spleen, edema and urine-stone in the kidney, turbid swelling and small hemorrhage in the heart were observed. Moreover there were a decrease of glycogen, RNA and sometimes DNA in the liver.

3. Hematologic findings showed slight anemia, severe leucopenia with granulocytopenia and a little decrease of the blood platelet count.

4. Value of folic acid was decreased in the whole blood, liver and kidney, more remarkably in the urine.

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# HISTOCHEMICAL STUDIES ON MONOAMINE OXIDASE (MAO) OF THE HUMAN RETINA

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The histochemical researches on the MAO of the human retina will be demonstrated in the present report.

## Methods

After the enucleated eye-cups were incubated at 37°C for 60 minutes in the following mixture, they were fixed in 10% formol and studied in frozen sections. This mixture contained 25 mg of tryptamine hydrochloride, 4 mg. of sodium sulfate, 5 ml. of 0.1 M. phosphate buffer (pH 7.6), 15 ml. of distilled water and 20 mg. of neotetrazolium. Besides these ingredients, blue-tetrazolium and nitro-blue-tetrazolium respectively were used instead of neotetrazolium, and serotonin was applied as substrate instead of tryptamine hydrochloride.

## Results

1. By tryptamine-neotetrazolium method diformazan was recognised only very thinly in the upper layers of the corneal epithelium. The MAO was recognised in the sphincter of the iris, ciliary muscle (Fig. 1,2), non-pigmented epithelium of the iris, epithelium of the ciliary body (Fig. 3), and these activities were inhibited by 0.5 M hydroxylamine.

In the retina the MAO was found in the nerve fiber layer, ganglion cell layer, nuclear layers, outer reticular layer, visual cell layer and particularly in a pronounced degree in the Müller's fibers throughout the inner layers and outer reticular layer (Fig. 4). The in-vitro experiments showed that the MAO-active zones by the above-mentioned method became negative when 0.5 M hydroxylamine was added, and also that the MAO in the Müller's fibers and the ciliary muscle disappeared or declined generally in the outer zones when 0.5 M phenylhydrazine was added.

2. By tryptamine-bluetetrazolium method the MAO was strongly active in the innermost zone and was not observed in the Müller's fibers. Diformazan was thin and diffuse

Illustration of Figures.

Fig. 1. MAO in the Ciliary Body
(Tryptamine-NT)

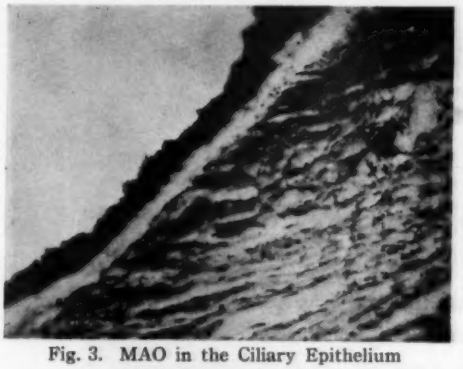


Fig. 2. MAO in the Ciliary Body
(Tryptamine-NT)

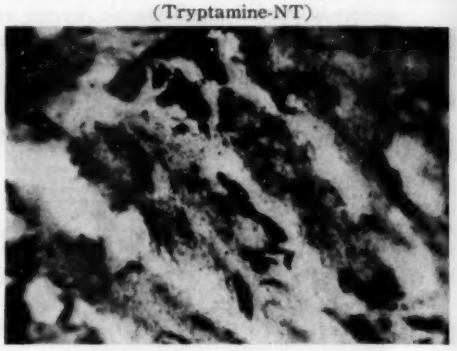


Fig. 3. MAO in the Ciliary Epithelium
(Tryptamine-NT)

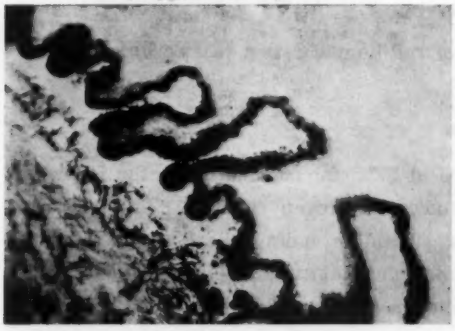


Fig. 4. MAO in the Retina
(Tryptamine-NT)

Fig. 5. MAO in the Retina
(Tryptamine-NBT)

Fig. 6. MAO in the Retina
(Tryptamine-NBT)

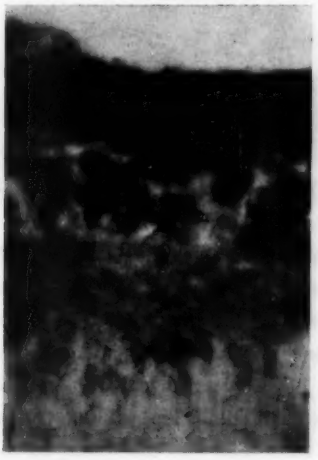


Fig. 7. MAO in the Edematous Retina Caused by the Orbital Tumor (Tryptamine-NBT)

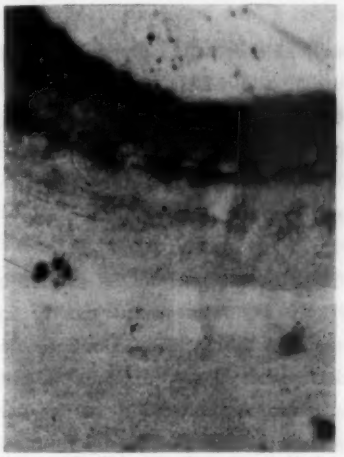


Fig. 8. MAO in the Traumatic Retinal Detachment (Tryptamine-NT)

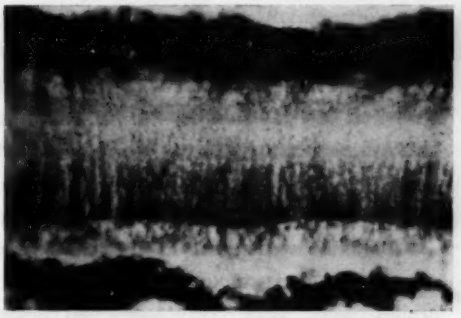


Fig. 9. MAO in the Traumatic Retinal Detachment (Tryptamine-NT)

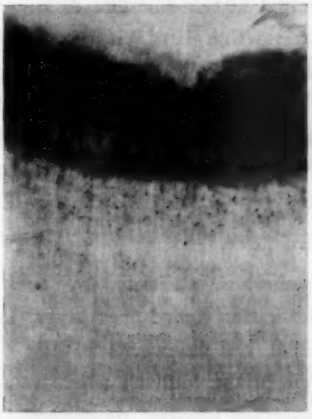


Fig. 10. MAO in the Traumatic Retinal Detachment (Serotonin-NT)

Fig. 11. MAO in the Edecatous Retina Caused by the Orbital Tumor (Serotonin-NBT)

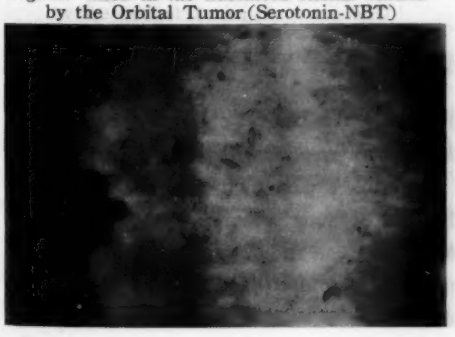
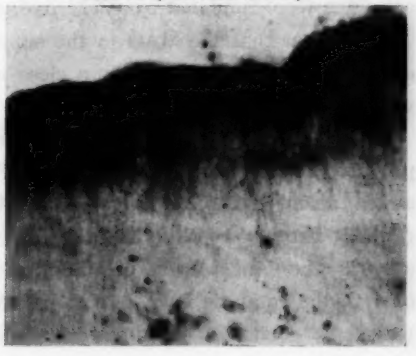


Fig. 12. MAO in the Retina with the Vitreous Abscess (Serotonin-NBT)



in the nuclear layers and monoformazan was recognised in the inner area of the reticular layers.

3. With tryptamine-nitrobluetetrazolium, Glenner's method was applied with 20 mg. instead of 5 mg. of Glenner's reagent. By this method the MAO activity was proved strong in the ganglion cells, amacrine cells, inner reticular layer, visual cell layer (Fig 5,6) and in some cases appeared throughout all the layers and particularly strong in the inner layers.

4. With serotonin-NT the MAO activity was strong in the inner layers.

5. With serotonon-NBT it was observed in the inner layers and visual cells.

As to the pathological materials, in the case with the retinal edema caused by an orbital tumor, under tryptamine-NBT preparation, the MAO was found mostly in the inner layers and to some extent in evry layer (Fig. 7). In the traumatic retinal detachment, under tryptamine-NT preparation, the MAO declined or disappeared in the supporting fibers (Fig. 8) and was recognised only in the inner layers (Fig. 9). In the case of staphyloma, the MAO was negative in the supporting fibers and existed only in the inner layers. In the traumatic eye which was prepared with serotonin-NT the MAO was very scarce or negative in the outer layers and was found in the dust-like diformazan in the ganglion cells, nerve fiber layer and nuclear layers (Fig. 10). With serotonin-NBT the MAO showed a decrease either in the ganglion cells or in the visual cells in the case of the retinal edema caused by the orbital tumor (Fig. 11), while it disappeared in the iris and ciliary body but remained slightly in the inner layers in the case of the vitreous abscess (Fig. 12).

## Discussion

The pattern of the MAO activity in the retina is generally elucidated by the tryptamine-NT method. The catalytic field of the MAO existed in the nerve fiber layer, ganglion cells, nuclear layers, outer reticular layer and the visual cell layer, as in the case of succinic dehydrogenase. With this method, the MAO in the Müller's fibers showed an evident increase throughout the inner layers and external limiting membrane. These activities were inhibited by 0.5 M hydroxylamine.

The fact that the MAO in the supporting fibers has connection with that in the visual cells shows that the MAO has a close interrelation with that of the inner segment of the visual cells.

The amino acids found in the retina suggest the presence of precursing amines produced by anerobic decarboxylation. The MAO goes in parallel with the catalytic field of amino acids in the retina indicating that the MAO takes part in the detoxication in the retina. The blood vessels are highly developed in the human retina, while they existed only in the inner layers in the case of a rabbit. In the rabbit retina the MAO exists only in the catalytic field, but not in the Müller's fibers, and it appears here only under pathologic conditions. On the contrary, the MAO is always active in the Müller's fibers

in the human retina. This fact may be related to the different distribution of the vascular system in the human and rabbit retinas, and the MAO is necessary for detoxicating the toxic agents put out of the blood vessels and participates in such functions of monoamines as may cause vascular constriction and dilatation, and moreover, the high activity of the MAO in Müller's fibers is necessiated for keeping these functions. The MAO activity is strong also in the ganglion cells and inner area of the supporting fibers, indicating the possible relationship to the concentration gradient of the monoamines in the inner layers.

While the MAO in the retina is found in the supporting fibers, nerve fiber layer, ganglion cells, nuclear layers and visual cells as a catalytic field, choline esterase is strongly active in the nerve fiber layer, outer nuclear layer and visual cells. The field of monoamine and acetylcholine as chemical conductive agents are inter-relating each other in the retina.

The histochemical demonstration of the MAO is excellently performed by tryptamine-NT method as shown above, Glenner also showed the mehtod with tryptamine-NBT. According to this Glenner's method, the MAO is well demonstrated in the ganglion cells, amacrine cells and inner segment, however it is not clear in the supporting fibers and the results are variable. With serotonin as substrated the MAO is strong in the inner layers by serotonin-NBT method. Thus, the tryptamine-NT method is adequate for demonstrating the whole pattern of the MAO and that in the supporting fibers and Glenner's method is recommendable for the study of the MAO in the ganglion cells, amacrine cells and inner segments. As to pathological materials, the inhibition of the MAO (tryptamine-NBT) in the outer layers in the case of the edematous retina, the declination of the MAO in the supporting fibers and disappearance in the outer layers (tryptamine-NT) are recognised in the retina of traumatic detachment and that of staphyloma. The MAO is inhibited either in the ganglion cells or in the visual cells (serotonin-NBT) in the case of the retinal edema by the orbital tumor. In the eye of trauma the MAO decreased in the outer layers (serotonin-NT), while in the case of the vitreous abscess, the disappearance of the MAO is observed in the outer layers, iris and ciliary body. As shown above, the originrl pattern of the MAO was proved to be inhibited in these pathologic materials.

## Conclusion

The histochemical studies on the MAO of the human retina, iris, ciliary body and cornea have been demonstrated in the present report.

1. By tryptamine-NT method the MAO was observed in the ganglion cells, nerve fiber layer, nuclear layers and visual cells in parallel with the catalytic field. Especially, the Müller's supporting fibers displayed an evident pattern of the MAO from the inner layers to the external limiting membrane by this method.

2. The MAO activity was strong in the ganglion cells, inner segment of the visual

cells by tryptamine-NBT method and was strong generally in the visual cells by serotonin-NBT method, but was less clearly demonstrated than by tryptamine-NT method.

3. By tryptamine-NT method the MAO was observed in the sphincter muscle and ciliary body, while in the cornea it was found only in the upper layer of the corneal epithelium and diformazan was not clear in the endothelial cells and parenchym.

4. The MAO in the retina has a close relation to the amine metabolism, detoxication mechanism, and stimulus conduction, as a catalytic field. Advanced degree of the MAO activity in the supporting fibers indicates a deep connection with the vascular distribution in these fibers and with the concentration gradient of the amines.

5. With the retinas of trauma and stapyloma, the MAO activity showed a decrease in the outer layers (tryptamine-NBT) in the inner layers or in the outer layers (serotonin-NBT), and in the retina of the vitreous abscess it disappeared in the outer layers (serotonin-NBT), thus, the resistance of the MAO activity is generally weak in the outer layers. The presence of the MAO in the retina confirms that there is a metabolic field of tryptamine and serotonin in the retina.

6. The presence of the MAO in the sphincter muscle, ciliary muscle and epithelium of the iris and ciliary body may indicate that it might take part in the accommodation and detoxication mechanism.

7. In the corneal epithelium the MAO existed only in the upper layer.

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## RELATIONSHIP BETWEEN BLOOD-WATER CONTENT AND DIURNAL VARIATION OF THE INTRAOCULAR PRESSURE

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In the previous paper, the author reported the experimental results regarding the relationship between the blood-water content and the intraocular pressure (IOP) in the water-drinking test and it was found that there was no parallelism between the two. Most of the cases tested exhibited an apparent decrease in blood-water content instead of elevated IOP induced by the test. This led the author to the concept, that it was not plausible to consider the elevation of IOP caused by the test to be the effect of decreased osmotic blood pressure induced by hemodilution after water drinking and that

it seemed justified to believe, that the water drinking itself should have been responsible for the elevation of IOP, the water drinking having been followed by any change in autonomic nervous system which in turn effected an elevation of IOP.

That IOP and blood-water content show diurnal variations is well known and a decrease in osmotic blood pressure after water drinking has been widely supposed to give rise to an elevation of IOP. The diurnal variations of IOP have been, therefore, generally attributed to the change in osmotic blood pressure. The author's concept from his previous report doesn't agree with this explanation. It is the purpose of the present report to confirm his concept in case of diurnal variation of IOP.

## Methods

Five normal subjects and ten glaucoma patients, seven of primary and three of secondary type were used for the experiments. All were hospitalized and IOP was measured with Schiötz's tonometer under topical anesthesia with nupercaine (0.3%). Simultaneously with the tonometric measurement blood was taken from the median cubital vein for estimation of blood-water content by means of Kuroda's method. These procedures were performed every three hours from 6 : 00 a.m. to 9 : 00 p.m., in some cases every two hours until 10 : 00 p.m. Meals were given twice a day, at 7 : 00 a.m. and 6 : 00 p.m., in order to minimize the influence of taking foods upon the blood and IOP and to keep the subjects not too hungry. They were kept in bed all through the period of procedures but radio and talks were allowed to prevent them from falling asleep or feeling drowsy.

## Results

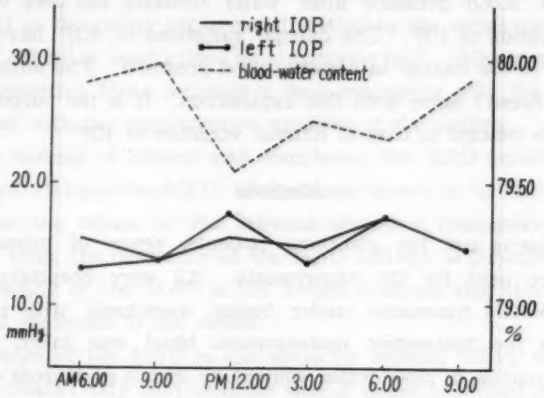
In the normal subjects, the difference between maximum and minimum values of IOP in the period from 6 : 00 a.m. to 9 : 00 p.m. ranged from 1.7 to 4.3 mmHg., with a mean value of 2.5 mmHg. No apparent type of diurnal variation was found, except one case of falling type.

The difference between maximum and minimum values of blood-water content in all five cases ranged from 0.37 to 1.04%, with a mean value of 0.77%. As to the type of the diurnal variation, two cases showed gradual decrease, one case gradual increase toward evening, and in the remaining two cases, the blood-water content decreased gradually to reach the minimum value at noon, increasing in turn thereafter. In regard to the relation between IOP and blood-water content, each is expected to have a period of maximum and minimum value but there was no coincidental minimum value period found. In two cases, No. 4 and 5, the maximum value period of IOP coincided with that of blood-water content while in one case, No. 1, the minimum value period of IOP coincided with the maximum one of blood-water content, and in case 2, on the contrary, the maximum value period of IOP coinciding with the minimum one of blood-water content. Furthermore, as is shown in Fig. 1 (case 3) exhibited an exact inverse re-

lationship of the variation of IOP and blood-water content.

After all, there was no parallel relationship found between the variations of IOP and blood-water content.

Fig. 1. Normal Subject No. 3




In the glaucomatous patients, measurements were taken every three hours from 6:00 a.m. to 9:00 p.m. in three cases, every two hours from 6:00 a.m. to 10:00 p.m. in seven cases, in total 17 glaucomatous eyes were included as there were some patients with only one eye affected. In these 17 glaucomatous eyes, the difference between maximum and minimum values of IOP ranged from 5.1 to 19.2 mmHg., with a mean value of 11.5 mmHg. As for the type of diurnal variation of IOP, there were three cases of double variation type, three of falling and four of the rising type. The difference between maximum and minimum values of blood-water content was from 0.33 to 1.4%, 0.71% in average.

As to the type of diurnal variation of blood-water content, there were five cases with a tendency to gradual decrease, and two with that to gradual increase and another three had no definite type of variation. The maximum value period of IOP and blood-water content was coincidental in three cases and the minimum one in two cases. Both the maximum and the minimum value periods coincided respectively in only one case. On the contrary, the blood-water content gave maximum value when IOP gave the minimum value, in five cases. After all, in the case of glaucoma patients, the variation of IOP and blood-water content showed parallelism only in one case and in the other nine cases there was no parallel relation found.

## Discussion

Though no measurement was taken in the tests while the subjects were asleep, it has been already reported by some authors, that increase in blood-water content occurs during sleep or even by sleepiness, tending to decrease with awakening. It is expected that the

IOP might be influenced by sleep or sleepiness, although no definite data are available. Therefore, to avoid any factors to implicate the experiment, care was taken not to let the subjects fall asleep or to keep them awake.

As to the diurnal variation of blood-water content, of 15 subjects, composed of glaucomatous and normal, seven cases showed gradual decrease from early morning toward the end of the test period in the evening and the remaining eight cases showed no such tendency, of whom three showed variation in the reverse manner. Despite of every caution, the gradual diurnal decrease of blood-water content might have been the result from sleepiness of the subjects, because they were kept quiet without any activity all through the period of examinations.

Between the normal subjects and the glaucomatous patients, there was no difference in regard to the amount of the diurnal variation of blood-water content.

Next, it has long been well-known that the IOP has a diurnal variation and that its amount is larger in glaucoma cases than in normal eyes, the feature that is used generally in diagnosis of glaucoma. The results obtained in these experiments agree with this notion, the variation in the normal subjects being 1.7-4.3 mmHg., in the mean 2.5 mmHg., while in the glaucoma patients 5.1-19.2 mmHg., in the mean 11.5 mmHg. The type of diurnal variation of IOP had been generally admitted, since Köllner, Thiel and others, to be falling type, i.e. with the highest value in the early morning and the lowest one in the evening. In 1951, however, Langley and Swanljung objected to this concept, classifying the variation into 4 types, double variation type, rising type, falling type and flat type. Since then this classification has been ascertained by Duke-Elder, Kishimoto and others.

According to the results in the present experiments, the falling type was found in one case (two eyes) among five (ten eyes) of normal subjects with smaller range of diurnal variation of the IOP. In other four normal subjects (eight eyes) no definite type of variation was detected.

Among ten glaucoma patients, only three cases showed a falling type and the other three (four eyes) showed the double variation type and the remaining four (seven eyes) showed the rising type. According to the results presented by Langley and Swanljung, the double variation type was dominant (more than one half of all), but Kishimoto stated that the falling type was popular (one third of all), the rising and the double variation type having been less frequent, a circumstance also seen in this experiment.

As to the cause of such diurnal variation of the IOP, Duke-Elder enumerated various possible factors (changes in the blood pressure, osmotic changes in the blood, action of light upon the ocular capillaries, size or movement of the pupil, massage of the eye by muscular movement, etc.) and stated that none of them had reliable correlation with the diurnal variation of IOP and that, even if the habits of meals, work and rest were changed, which were all supposed to cause osmotic changes in the blood, the rythm of the IOP variation was not caused to change itself. On the other hand, Shimizu, measur-

ing diurnal variation of colloidal osmotic pressure of the blood in normal subjects, found that it was decreased in the midnight during the early morning hours, from 2 a.m. till 5 : 00 a.m. Though he himself had taken no measurement of IOP, he conceived, referring to the general conception of the higher level of pressure in these hours, that there was a close relationship between the two and that the variation of IOP was subject to the changes of colloidal osmotic pressure of the blood, while the latter was under the control of the state of hypophamine secretion. Though this aspect of the colloidal osmotic pressure change cannot be denied when inferred from the known diurnal variation of the blood-water content, it will go too far to affirm directly that it coincides with the cause and aspect of the diurnal variation of the IOP, because the type of the diurnal variation of the IOP is not necessarily a falling type. The results obtained by Langley, Duke-Elder, Kishimoto and others in glaucoma and by Erickson and others in normal subjects should be considered in this respect. Erickson stated in his report of diurnal variation of the IOP in normal subjects: IOP takes its maximum value around from 8 : 00 a.m. to the noon, decreases gradually thereafter to take a minimum one at midnight and again begins to increase gradually from about 4 : 00 a.m. Accordingly, in order to observe an influence of osmotic pressure of the blood on diurnal variation of the IOP, it should be required to measure the two simultaneously in the same subject.

In the present experiment, the author aimed to elucidate the influence of the osmotic pressure change of the blood upon the diurnal variation of the IOP, measuring the blood-water content simultaneously with that of the IOP. Against expectation, among 15 cases including normal subjects and glaucoma patients, only one case showed a parallel relationship between the two. Even if it was excluded to take account of the individual value, comparing ronughly the general tendencies of the variations, coincidence was found in only three cases among all, whereas in four cases more or less reverse relationship was seen.

From the results presented in this report, it is to be concluded, that there is no definite relationship between diurnal variation of blood-water content, and of IOP. Though the cause of the diurnal variation of the IOP remains still unknown, there are many who suppose it to be attributed to the control of the nervous center, and Duke-Elder, as mentioned above, is one of them. Weinstein and Forgacs think of it from the relationship confirmed between cubital venous pressure and IOP, and Stepanik thinks of some direct effects of hypophysis on IOP, observing a correlation between IOP and a variation of the eosinophilic leucocyte count in the circulating blood. These views have not yet been wholly recognized, but, at the least, it is impossibtoconceive that change of osmotic pressure of the blood has an important effect on diurnal variation of the IOP.

## Summary

Simultaneous measurements of IOP and blood-wate content at regular intervals from early morning up to bedtime have been carried out on five normal subjects and

ten glaucoma patients. It was revealed that there were no relationship between IOP and blood-water content observable. In only one case of the total 15 a parallel diurnal variation was shown and in some cases a more or less reverse relationship between the two was seen.

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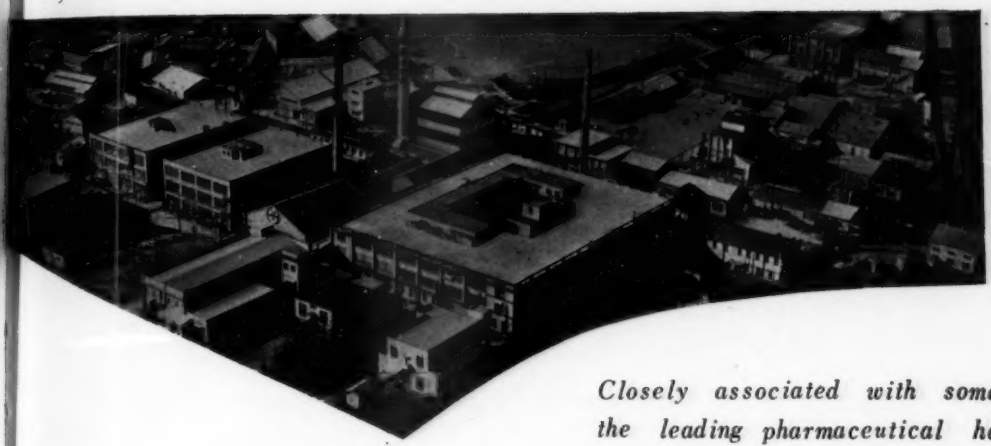

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